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APPLICATION

FOR

UNITED STATES LETTERS PATENT

TITLE: NUCLEIC ACIDS ENCODING ANTHELMINTIC AGENTS AND PLANTS MADE THEREFROM

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Nucleic Acids Encoding Anthelmintic Agents And Plants Made Therefrom

This application claims priority to U.S. Provisional Application No. 60/445,293, filed February 5, 2003, which is incorporated by reference in its entirety.

FIELD OF THE INVENTION

This invention relates to the field of plant pathology and plant genetic transformation. More particularly, the invention relates to methods and compositions for controlling plant pathogens such as plant-parasitic nematodes.

BACKGROUND OF THE INVENTION

Nematodes (derived from the Greek word for thread) are active, flexible, elongate, organisms that live on moist surfaces or in liquid environments, including films of water within soil and moist tissues within other organisms. While only 20,000 species of nematode have been identified, it is estimated that 40,000 to 10 million actually exist. Some species of nematodes have evolved to be very successful parasites of both plants and animals and are responsible for significant economic losses in agriculture and livestock and for morbidity and mortality in humans (Whitehead (1998) *Plant Nematode Control.* CAB International, New York).

Nematode parasites of plants can inhabit all parts of plants, including roots, developing flower buds, leaves, and stems. Plant parasites are classified on the basis of their feeding habits into the broad categories: migratory ectoparasites, migratory endoparasites, and sedentary endoparasites. Sedentary endoparasites, which include the root knot nematodes (*Meloidogyne*) and cyst nematodes (*Globodera* and *Heterodera*) induce feeding sites and establish long-term infections within roots that are often very damaging to crops (Whitehead, *supra*). It is estimated that parasitic nematodes cost the horticulture and agriculture industries in excess of $78 billion worldwide a year, based on an estimated average 12% annual loss spread across all major crops. For example, it is estimated that nematodes cause soybean losses of approximately $3.2 billion annually worldwide (Barker et al. (1994) *Plant and Soil Nematodes: Societal Impact and Focus for the Future.* The Committee on National Needs and Priorities in Nematology. Cooperative State Research Service, US Department of Agriculture and Society of Nematologists). Several factors make

the need for safe and effective nematode controls urgent. Continuing population growth, famines, and environmental degradation have heightened concern for the sustainability of agriculture, and new government regulations may prevent or severely restrict the use of many available agricultural anthelmintic agents.

The application of chemicals nematicides remains the major means of nematode control. However, in general, chemical nematicides are highly toxic compounds known to cause substantial environmental impact and are increasingly restricted in the amounts and locations in which then can be used. For example, the soil fumigant methyl bromide which has been used effectively to reduce nematode infestations in a variety of these specialty crops, is regulated under the U.N. Montreal Protocol as an ozone-depleting substance and is scheduled for elimination in 2005 in the US (Carter (2001) *California Agriculture*, 55(3):2). It is expected that strawberry and other commodity crop industries will be significantly impacted if a suitable replacement for methyl bromide is not found. Similarly, broad-spectrum nematicides such as Telone (various formulations of 1,3-dichloropropene) have significant restrictions on their use because of toxicological concerns (Carter (2001) *California Agriculture*, Vol. 55(3):12-18).

The macrocyclic lactones (e.g., avermectins and milbemycins), as well as delta-endotoxins from *Bacillus thuringiensis* (*Bt*), are chemicals that in principle provide excellent specificity and efficacy which should allow environmentally safe control of plant parasitic nematodes. Unfortunately, in practice, these two nematicidal agents have proven less effective in agricultural applications against root pathogens. Although certain avermectins show exquisite activity against plant parasitic nematodes these chemicals are hampered by poor bioavailability due to their light sensitivity, tight binding to soil particles and degradation by soil microorganisms (Lasota & Dybas (1990) *Acta Leiden* 59(1-2):217-225; Wright & Perry (1998) Musculature and Neurobiology. In: The Physiology and Biochemistry of Free-Living and Plant-parasitic Nematodes (eds R.N. Perry & D.J. Wright), CAB International 1998). Consequently despite years of research and extensive use against animal parasitic nematodes, mites and insects (plant and animal applications), macrocyclic lactones (e.g., avermectins and milbemycins) have never been commercially developed to control plant parasitic nematodes in the soil.

Bt delta endotoxins must be ingested to affect their target organ, the brush border of midgut epithelial cells (Marroquin et al. (2000) *Genetics*. 155(4):1693-1699). Consequently they are not anticipated to be effective against the dispersal, non-feeding, juvenile stages of plant parasitic nematodes in the field. Because juvenile stages only commence feeding when a susceptible host has been infected, nematicides may need to penetrate the plant cuticle to be effective. Transcuticular uptake of a 65-130 kDa protein - the size of typical *Bt* delta ends toxins - is unlikely. Furthermore, soil mobility is expected to be relatively poor. Even transgenic approaches are hampered by the size of *Bt* delta toxins because delivery *in planta* is likely to be constrained by the exclusion of large particles by the feeding tubes of certain plant parasitic nematodes such as *Heterodera* (Atkinson et al. (1998) Engineering resistance to plant-parasitic nematodes. In: The Physiology and Biochemistry of Free-Living and Plant-parasitic Nematodes (eds R.N. Perry & D.J. Wright), CAB International 1998).

Fatty acids are another class of natural compounds that have been investigated as alternatives to the toxic, non-specific organophosphate, carbamate and fumigant pesticides (Stadler et al. (1994) *Planta Medica* 60(2):128-132; US Pat. Nos. 5,192,546; 5,346,698; 5,674,897; 5,698,592; 6,124,359). It has been suggested that fatty acids derive their pesticidal effects by adversely interfering with the nematode cuticle or hypodermis via a detergent (solubilization) effect, or through direct interaction of the fatty acids and the lipophilic regions of target plasma membranes (Davis et al. (1997) *Journal of Nematology* 29(4S):677-684). In view of this predicted mode of action it is not surprising that fatty acids are used in a variety of pesticidal applications including as herbicides (e.g., SCYTHE by Dow Agrosciences is the C9 saturated fatty acid pelargonic acid), bactericides and fungicides (US Pat. Nos. 4,771,571; 5,246,716) and insecticides (e.g., SAFER INSECTICIDAL SOAP by Safer, Inc.).

The phytotoxicity of fatty acids has been a major constraint on their general use in post-plant agricultural applications (US Pat. No. 5,093,124) and the mitigation of these undesirable effects while preserving pesticidal activity is a major area of research. Post-plant applications are desirable because of the relatively short half-life of fatty acids under field conditions.

The esterification of fatty acids can significantly decrease their phytotoxicity (US Pat. Nos. 5,674,897; 5,698,592; 6,124,359). Such modifications can however lead to loss of

nematicidal activity as is seen for linoleic, linolenic and oleic acid (Stadler et al. (1994) *Planta Medica* 60(2):128-132) and it may be impossible to completely decouple the phytotoxicity and nematicidal activity of pesticidal fatty acids because of their non-specific mode of action. Perhaps not surprisingly, the nematicidal fatty acid pelargonic acid methyl ester (US Pat. Nos. 5,674,897; 5,698,592; 6,124,359) shows a relatively small "therapeutic window" between the onset of pesticidal activity and the observation of significant phytotoxicity (Davis et al. (1997) *J Nematol* 29(4S):677-684). This is the expected result if both the phytotoxicity and the nematicidal activity derive from the non-specific disruption of plasma membrane integrity.

Ricinoleic acid, the major component of castor oil, has been shown to have an inhibitory effect on water and electrolyte absorption using everted hamster jejunal and ileal segments (Gaginella et al. (1975) *J Pharmacol Exp Ther* 195(2):355-61) and to be cytotoxic to isolated intestinal epithelial cells (Gaginella et al. (1977) J Pharmacol Exp Ther 201(1):259-66). These features are likely the source of the laxative properties of castor oil which is given as a purgative in humans and livestock (e.g., castor oil is a component of some de-worming protocols because of its laxative properties). In contrast, the methyl ester of ricinoleic acid is ineffective at suppressing water absorption in the hamster model (Gaginella et al. (1975) *J Pharmacol Exp Ther* 195(2):355-61).

It has been reported that short- and medium-chain fatty acids and salts (e.g., C6 to C12) have superior fungicidal activity (US Pat. Nos. 5,093,124 and 5,246,716). Not surprisingly, the commercial fungicidal and moss killing product De-Moss comprises mainly fatty acids and salts in this size range. The phytotoxicity of these shorter fatty acids also makes them suitable as broad-spectrum herbicides when used at higher concentrations as is exemplified by the commercial herbicide SCYTHE which comprises the C9 fatty acid pelargonic (nonanoic) acid. US Pat. Nos. 5,093,124, 5,192, 546, 5,246,716 and 5,346,698 teach that C16 to C20 fatty acids and salts such as oleic acid (C18:1) are suitable insecticidal fatty acids. Insecticidal fatty acid products such as M-PEDE and SAFER Insecticidal Concentrate whose active ingredients comprise longer chain fatty acids rich in C16 and C18 components represent real world applications of this scientific information. In contrast, the prior art provides little guidance for the selection of suitable broad-spectrum nematicidal fatty acids and what information exists is often contradictory.

Stadler and colleagues (Stadler et al. (1994) *Planta Medica* 60(2):128-132) tested a series of fatty acids against L4 and adult *C. elegans* and found that a number of common longer chain fatty acids such as linoleic (C18:2), myristic (C14:0), palmitoleic (C16:1) and oleic (C18:1) acids had significant nematicidal activity. *C. elegans* was not very sensitive to C6 to C10 (medium chain) fatty acids. Stadler et al. commented that their results contrasted with those of an earlier study on the plant parasite *Aphelenchoides besseyi* where C8 to C12 fatty acids were found to be highly active while linoleic acid – a C18 fatty acid – showed no activity. The differential sensitivity of specific nematodes to various fatty acids is again evident in the study of Djian and co-workers (Djian et al. (1994) *Pestic. Biochem. Physiol.* 50(3):229-239) who demonstrate that the nematicidal potency of short volatile fatty acids such as pentanoic acid can vary between species (e.g., *Meloidogyne incognita* is over a hundred times more sensitive than *Panagrellus redivivus*). The recent finding by Momin and Nair (Momin & Nair (2002) *J. Agric. Food Chem.* 50(16):4475-4478) that oleic acid at 100 μg/mL over 24 hours is not nematicidal to either *Panagrellus redivivus* or *Caenorhabditis elegans* further confuses the situation as it directly conflicts with the LD50 of 25 μg/mL (LD90 100 μg/mL) measured by Stadler and coworkers.

In summary, unlike the case for fungicides, herbicides and insecticides, the prior art provides no specific or credible guidance to aid in the selection of suitable nematicidal fatty acids. Moreover, whereas De-Moss, SCYTHE, M-PEDE and SAFER, are examples of successful pesticidal fatty acid products in these three areas respectively, there are currently no examples of commercial nematicidal fatty acid products in widespread use.

Many plant species are reported to be highly resistant to nematodes. The best documented of these include marigolds (*Tagetes* spp.), rattlebox (*Crotalaria spectabilis*), chrysanthemums (*Chrysanthemum* spp.), castor bean (*Ricinus communis*), margosa (*Azardiracta indica*), and many members of the family *Asteraceae* (family *Compositae*) (Hackney & Dickerson. (1975) *J Nematol* 7(1):84-90). In the case of the *Asteraceae*, the photodynamic compound alpha-terthienyl has been shown to account for the strong nematicidal activity of the roots. Castor beans are plowed under as a green manure before a seed crop is set. However, a significant drawback of the castor plant is that the seed contains toxic compounds (such as ricin) that can kill humans, pets, and livestock and is also highly allergenic. In many cases however, the active principle(s) for plant nematicidal activity has

not been discovered and it therefore remains difficult to derive commercially successful nematicidal products from these resistant plants or to transfer the resistance to agronomically important crops such as soybeans and cotton.

Genetic resistance to certain nematodes is available in some commercial cultivars (e.g., soybeans), but these are restricted in number and the availability of cultivars with both desirable agronomic features and resistance is limited. The production of nematode resistant commercial varieties by conventional plant breeding based on genetic recombination through sexual crosses is a slow process and is often further hampered by a lack of appropriate germplasm.

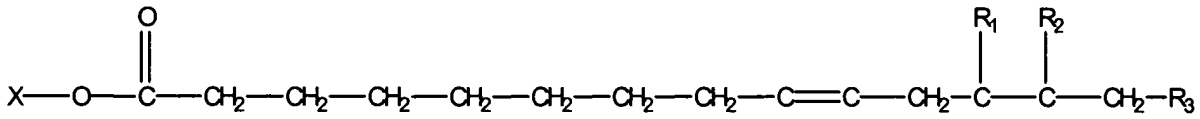
Small chemical effectors can have significant advantages where size exclusion of larger molecules is a concern (e.g., with sedentary plant parasitic nematodes). However, unless the small molecule nematicidal active has high in planta mobility, or the chemical stimulates increased systemic resistance, a transgene encoding an enzyme must still be expressed in an appropriate spatial and temporal manner to be effective. With many plant parasitic nematodes this means that root expression of the nematicidal product is likely important for nematode control. It has been reported that when a constitutive promoter such as a Cauliflower Mosaic Virus (CaMV) *35S* promoter is used to drive expression of certain hydroxylase enzymes, no significant amounts of protein production or hydroxylase activity is observed in non-seed tissues (e.g., roots or leaves), nor do hydroxylated fatty acids accumulate (van de Loo et al. (1995) *Proc Natl Acad Sci U S A* 92(15):6743-7; Broun & Sommerville (1997) *Plant Physiol.* 113(3):933-942; Broun et al. (1998) *Plant J.* 13(2):201-210; US 6,291,742; US 6,310,194).

There remains an urgent need to develop environmentally safe, target-specific ways of controlling plant parasitic nematodes. In the specialty crop markets, economic hardship resulting from nematode infestation is highest in strawberries, bananas, and other high value vegetables and fruits. In the high-acreage crop markets, nematode damage is greatest in soybeans and cotton. There are however, dozens of additional crops that suffer from nematode infestation including potato, pepper, onion, citrus, coffee, sugarcane, greenhouse ornamentals and golf course turf grasses.

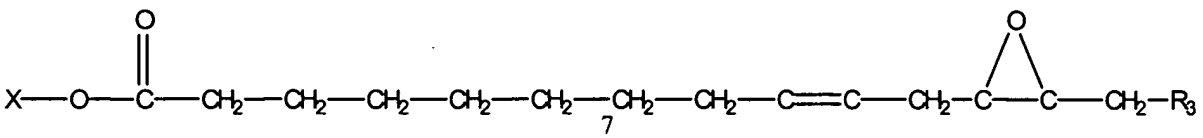
SUMMARY OF THE INVENTION

The invention concerns DNA constructs that include sequences encoding fatty acid hydroxylases or epoxygenases, transgenic plants harboring such constructs, and methods for making such transgenic plants. These transgenic plants can exhibit increased resistance to nematodes and can be useful for controlling nematodes in an environmentally safe manner. The invention is based in part on the surprising discovery that certain hydroxylated or epoxygenated fatty acids and methyl esters (e.g., ricinoleate, vernolate), exhibit nematicidal activity. These fatty acids show significantly enhanced nematicidal activity over other eighteen carbon free fatty acids such as oleate, elaidate and linoleate. Nucleic acids encoding hydroxylase or epoxygenase polypeptides can be introduced into plants in order to increase the levels of hydroxylated or epoxygenated fatty acids and thus aid in controlling nematode damage in commercially important plant species.

In one aspect, the invention features a transgenic plant containing at least one DNA construct. The construct comprises at least one regulatory element that confers expression in vegetative tissues of a plant. The regulatory element is operably linked to a nucleic acid encoding a polypeptide that is effective for catalysing the conversion of a substrate to a C16, C18, or C20 monounsaturated fatty acid product. The C16-C20 monounsaturated fatty acid product can be:



wherein X is hydrogen, CoA, glycerol, a monoglyceride, a diglyceride, ACP, methyl, Na+, phosphatidylcholine, or phosphatidylethanolamine, wherein both R_1 and R_2 are hydroxyl, one of R_1 and R_2 is hydroxyl and the other is hydrogen, or one of R_1 and R_2 is keto and the other is hydrogen, and wherein R_3 is C2, C4, or C6 alkyl. The C16-C20 monounsaturated fatty acid product can also be:



wherein X is hydrogen, CoA, glycerol, a monoglyceride, a diglyceride, ACP, methyl, Na+, phosphatidylcholine, or phosphatidylethanolamine, and wherein R_3 is C2, C4, or C6 alkyl.

The C=C double bond can be *cis* or *trans*. The R_3 moiety of the C16-C20 monounsaturated fatty acid product can be C2 alkyl. A C16-C20 monounsaturated fatty acid product can have hydroxy, hydrogen, and C4 alkyl as the R_1, R_2 and R_3 moieties, respectively, e.g., a ricinoleate product. Alternatively, a C16-C20 monounsaturated fatty acid product can have an epoxy moiety at the 12^{th} and 13^{th} carbons counting from the carbonyl carbon and C4 alkyl at R_3, e.g., a vernolate product.

The plant can have an increased amount of a hydroxy-fatty acid, e.g., ricinoleic acid, in a vegetative tissue, relative to a corresponding plant that lacks the DNA construct. The hydroxy-fatty acid can constitute from about 0.01% to about 25% of the total fatty acid content of the tissue. In some embodiments, the plant has an increased amount of a epoxy-fatty acid, e.g., vernolic acid, in a vegetative tissue, relative to a corresponding plant that lacks the DNA construct. The epoxy-fatty acid can constitute from about 0.01% to about 35% of the total fatty acid content of the tissue.

The regulatory element can be a 5'-regulatory element or a 3'-regulatory element. The regulatory element can confer expression in root tissue, or in leaf tissue. For example, a 5'-regulatory element can be a CaMV*35S* promoter, a potato ribosomal protein S27a Ubi3 promoter, an alfalfa histone H3.2 promoter, an IRT2 promoter, an RB7 promoter, an *Arabidopsis* FAD2 5'-UTR, an *Arabidopsis* FAD3 5'-UTR, a Ubi3 5'-UTR, an alfalfa histone H3.2 5'-UTR, or a CaMV35S 5'-UTR.

There can be more than one regulatory element operably linked to the polypeptide coding sequence in the DNA construct. For example, a DNA construct can have two 5'-regulatory elements. The first 5'- regulatory element can be a Ubi3 promoter and the second 5'-regulatory element can be an *Arabidopsis* FAD2 5'-UTR, an *Arabidopsis* FAD3 5'-UTR, a potato ribosomal protein S27a Ubi3 5'-UTR, or a CaMV35S 5'-UTR. In some embodiments the DNA construct has a 5'-regulatory element and a 3'-regulatory element. The 3'-regulatory element can be a Ubi3 terminator or an E9 pea terminator. Alternatively, the 5'-regulatory element can be an *Arabidopsis* FAD2 5'-UTR or an *Arabidopsis* FAD3 5'-

UTR and the 3'-regulatory element can be an *Arabidopsis* FAD2 3'-UTR or an *Arabidopsis* FAD3 3'-UTR.

The DNA construct in a plant can include a nucleic acid that encodes a PDAT or DAGAT or lipase polypeptide, operably linked to one or more regulatory elements that confer expression in vegetative tissues of a plant. Alternatively, the PDAT or DAGAT or lipase coding sequence and regulatory element can be part of a separate DNA construct in the plant. In some embodiments, the plant contains a DNA construct encoding a delta–12 or delta–15 fatty acid desaturase.

The amino acid sequence of the polypeptide can be SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, *C. palaestina* epoxygenase GenBank® No. CAA76156, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, a *C. palaestina* epoxygenase chimera, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41 or SEQ ID NO: 42. The nucleic acid encoding the polypeptide can be SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, or SEQ ID NO: 33.

The plant can be a monocotyledonous or a dicotyledonous plant. For example, the plant can be a soybean, corn, cotton, rice, tobacco, tomato, wheat, banana, carrot, potato, strawberry or turf grass plant.

In another aspect, the invention features a method of making a transgenic plant. The method comprises obtaining a DNA construct as described herein, and introducing the construct into a plant. The DNA construct can include nucleic acids encoding the polypeptides described herein, and can include the regulatory elements described herein.

The invention also features a method of screening a transgenic plant for anthelmintic activity. The method comprises contacting a transgenic plant with a nematode under conditions effective to determine whether or not the plant has anthelmintic activity. For example, the nematodes can be contacted with one or more roots of the transgenic plant. The transgenic plant has a DNA construct that include nucleic acids encoding a hydroxylase or

epoxygenase polypeptide described herein, and can include the regulatory elements described herein. The method can also be carried out with plant tissue, e.g., root tissue, leaf tissue or stem tissue from such a transgenic plant.

In another aspect, the invention features an isolated nucleic acid. The nucleic acid can comprise the nucleotide sequence set forth in SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, or SEQ ID NO: 33.

In another aspect, the invention features a recombinant nucleic acid construct. The construct comprises at least one regulatory element that confers expression in vegetative tissues of a plant. The regulatory element is operably linked to a nucleic acid having the nucleotide sequence set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32 or SEQ ID NO: 33. The regulatory element can confer expression in, for example, roots or leaves. The regulatory element can be a 5'-regulatory element having the nucleotide sequence set forth in SEQ ID NO: 43 or SEQ ID NO: 44. The nucleic acid construct can further comprise a 3'-regulatory element having the nucleotide sequence set forth in SEQ ID NO: 45.

The invention also features a transgenic plant harboring a DNA construct. The construct comprises a nucleic acid encoding a fatty acid epoxygenase polypeptide or a fatty acid hydroxylase polypeptide, operably linked to a regulatory element conferring expression of the polypeptide in a vegetative tissue of the plant. The polypeptide can have the amino acid sequence of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, *C. palaestina* epoxygenase (GenBank® No. CAA76156), SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, or SEQ ID NO: 42.

The plant can have a significantly increased amount of a hydroxy-fatty acid, e.g., ricinoleic acid, in a vegetative tissue of the plant relative to a corresponding plant that lacks the DNA construct. The hydroxy-fatty acid can constitute from about 0.1% to about 10% of

the total fatty acid content of the tissue. In some embodiments, the plant has a significantly increased amount of a epoxy-fatty acid, e.g., vernolic acid, in a vegetative tissue of the plant relative to a corresponding plant that lacks the DNA construct. The epoxy-fatty acid can constitute from about 0.1% to about 10% of the total fatty acid content of the tissue.

A "purified polypeptide", as used herein, refers to a polypeptide that has been separated from other proteins, lipids, and nucleic acids with which it is naturally associated. The polypeptide can constitute at least 10, 20, 50, 70, 80 or 95% by dry weight of the purified preparation.

An "isolated nucleic acid" is a nucleic acid, the structure of which is not identical to that of any naturally occurring nucleic acid, or to that of any fragment of a naturally occurring genomic nucleic acid spanning more than three separate genes. The term therefore covers, for example: (a) a DNA which is part of a naturally occurring genomic DNA molecule but is not flanked by both of the nucleic acid sequences that flank that part of the molecule in the genome of the organism in which it naturally occurs; (b) a nucleic acid incorporated into a vector or into the genomic DNA of a prokaryote or eukaryote in a manner such that the resulting molecule is not identical to any naturally occurring vector or genomic DNA; (c) a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), or a restriction fragment; and (d) a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein. Specifically excluded from this definition are nucleic acids present in mixtures of different (i) DNA molecules, (ii) transfected cells, or (iii) cell clones in a DNA library such as a cDNA or genomic DNA library. Isolated nucleic acid molecules according to the present invention further include molecules produced synthetically, as well as any nucleic acids that have been altered chemically and/or that have modified backbones.

Although the phrase "nucleic acid molecule" primarily refers to the physical nucleic acid molecule and the phrase "nucleic acid sequence" refers to the sequence of the nucleotides in the nucleic acid molecule, the two phrases can be used interchangeably.

The term "substantially pure" as used herein in reference to a given polypeptide means that the polypeptide is substantially free from other biological macromolecules. The substantially pure polypeptide is at least 75% (e.g., at least 80, 85, 95, or 99%) pure by dry

weight. Purity can be measured by any appropriate standard method, for example, by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

The term "ectopic expression" refers to a pattern of subcellular, cell-type, tissue-type and/or developmental or temporal expression that is not normal for the particular gene or enzyme in question. It also refers to expression of a heterologous gene; e.g. a gene not naturally occurring in the organism (also termed "transgene" as described below). Such ectopic expression does not necessarily exclude expression in normal tissues or developmental stages.

As used herein, the term "transgene" means a nucleic acid that is partly or entirely heterologous, i.e., foreign, to the transgenic plant, animal, or cell into which it is introduced, or, is homologous to an endogenous gene of the transgenic plant, animal, or cell into which it is introduced, but which is inserted into the plant's genome in such a way as to alter the genome of the cell into which it is inserted (e.g., it is inserted at a location which differs from that of the natural gene or its insertion results in a knockout). A transgene can include one or more regulatory elements operably linked to a polypeptide coding sequence.

As used herein, the term "transgenic cell" refers to a cell containing a transgene. As used herein, a "transgenic plant" is any plant in which one or more, or all, of the cells of the plant includes a transgene. A transgene may be integrated within a chromosome, or it may be extrachromosomally replicating DNA.

The terms "operably linked", "operably inserted" or "operably associated" mean that a regulatory element is positioned in a DNA construct relative to a polypeptide coding sequence so as to effect expression of the polypeptide.

As used herein, the terms "hybridizes under stringent conditions" and "hybridizes under high stringency conditions" refers to conditions for hybridization in 6X sodium chloride/sodium citrate (SSC) buffer at about 45 °C, followed by two washes in 0.2 X SSC buffer, 0.1% SDS at 60 °C or 65 °C. As used herein, the term "hybridizes under low stringency conditions" refers to conditions for hybridization in 6X SSC buffer at about 45 °C, followed by two washes in 6X SSC buffer, 0.1% (w/v) SDS at 50 °C.

A "heterologous promoter", when operably linked to a nucleic acid sequence, refers to a promoter which is not naturally associated with the nucleic acid sequence.

As used herein, the term "binding" refers to the ability of a first compound and a second compound that are not covalently linked to physically interact. The apparent dissociation constant for a binding event can be 1 mM or less, for example, 10 nM, 1 nM, and 0.1 nM or less.

As used herein, the term "binds specifically" refers to the ability of an antibody to discriminate between a target ligand and a non-target ligand such that the antibody binds to the target ligand and not to the non-target ligand when simultaneously exposed to both the given ligand and non-target ligand, and when the target ligand and the non-target ligand are both present in molar excess over the antibody.

As used herein, the term "altering an activity" refers to a change in level, either an increase or a decrease in the activity, (e.g., an increase or decrease in the ability of the polypeptide to bind or regulate other polypeptides or molecules) particularly a fatty acid desaturase-like or fatty acid desaturase activity (e.g., the ability to introduce a double bond at the delta-12 position of a fatty acid). The change can be detected in a qualitative or quantitative observation. If a quantitative observation is made, and if a comprehensive analysis is performed over a plurality of observations, one skilled in the art can apply routine statistical analysis to identify modulations where a level is changed and where the statistical parameter, the p value, is, for example, less than 0.05.

Unless otherwise specified, a "substituted" carbon, carbon chain, or methyl, alkyl can have one or more hydrogens replaced by another group, e.g., a halogen or a hydroxyl group.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages

of the invention will be apparent from the description and drawings, examples and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a set of drawings depicting the structures of ricinoleic acid, ricinelaidic acid, 12-oxo-9(Z)-octadecenoic acid, 12-oxo-9(E)-octadecenoic acid, (12,13)-epoxy-trans-9-octadecenoic acid and vernolic acid. The numbering of the carbons is indicated with the carbonyl (carboxyl) carbon being carbon 1. R = OH (acid); OCH_3 (methyl ester); O^-Na^+ (sodium salt)

FIG 2 is an alignment of the sequences of the hydroxylase and epoxygenase polypeptides (SEQ ID NO: 13 to 24; 34 to 42) and *A. thaliana* (SEQ ID NO: 125), *B. napus* (SEQ ID NO: 126), *G. max* (SEQ ID NO:127) and *S. indicum* (SEQ ID NO:128) FAD2 delta-12 desaturase polypeptides (gi|15229956|ref|NP_187819.1, gi|8705229|gb|AAF78778.1, gi|904154|gb|AAB00860.1 and gi|8886726|gb|AAF80560.1 respectively).

FIG 3 is a schematic representation of transgenic epoxygenase and hydroxylase constructs. HA refers to the amino acid sequence YPYDVPDYA (SEQ ID NO: 129), which corresponds to residues 99-107 of human influenza virus hemagglutinin. LB and RB refer to the left and right borders, respectively, of an *Agrobacterium* T-DNA.

FIG 4 is a schematic representation of the plasmid pUCAP3.

FIG 5 is a schematic representation of the plasmid pUCAP4.

FIG 6 is a schematic representation of the plasmid pUCAP6.

DETAILED DESCRIPTION

The present invention describes genes and genetic constructs encoding polypeptides effective for producing small molecule chemicals that show surprising nematicidal activity. The nematicidal activity is due in part to selective inhibition of metabolic processes that appear to be essential to nematodes and either absent or non-essential in vertebrates and plants. The invention therefore provides urgently needed DNA constructs, transgenic plants and methods of making such plants for environmentally safe control of plant-parasitic nematodes.

Fatty Acids

Unsaturated fatty acids are essential to the proper functioning of biological membranes. At physiological temperatures, polar glycerolipids that contain only saturated fatty acids cannot form the liquid-crystalline bilayer that is the fundamental structure of biological membranes. The introduction of an appropriate number of double bonds (a process referred to as desaturation) into the fatty acids of membrane glycerolipids decreases the temperature of the transition from the gel to the liquid-crystalline phase and provides membranes with necessary fluidity. Fluidity of the membrane is important for maintaining the barrier properties of the lipid bilayer and for the activation and function of certain membrane bound enzymes. There is also evidence that unsaturation confers some protection to ethanol and oxidative stress, suggesting that the degree of unsaturation of membrane fatty acids has importance beyond temperature adaptation. Unsaturated fatty acids are also precursors of polyunsaturated acids (PUFAs) arachidonic and eicosapentaenoic acids in animals, which are important sources of prostaglandins. These molecules are local hormones that alter the activities of the cells in which they are synthesized and in adjoining cells, mediating processes in reproduction, immunity, neurophysiology, thermobiology, and ion and fluid transport.

The ability of cells to modulate the degree of unsaturation in their membranes is primarily determined by the action of fatty acid desaturases. Desaturase enzymes introduce unsaturated bonds at specific positions in their fatty acyl chain substrates, using molecular oxygen and reducing equivalents from NADH (or NADPH) to catalyze the insertion of double bonds. In many systems, the reaction uses a short electron transport chain consisting of NAD(P)H, cytochrome b5 reductase, and cytochrome b5, to shuttle electrons from NAD(P)H and the carbon-carbon single bond to oxygen, forming water and a double bond (C=C). Many eukaryotic desaturases are endoplasmic reticulum (ER) bound non-heme diiron-oxo proteins that contain three conserved histidine-rich motifs and two long stretches of hydrophobic residues. These hydrophobic alpha helical domains are thought to position the protein with its bulk exposed to the cytosolic face of the ER and to organize the active site histidines to appropriately coordinate the active diiron-oxo moiety.

While most eukaryotic organisms, including mammals, can introduce a double bond into an 18-carbon fatty acid at the Δ9 position, mammals are incapable of inserting double

bonds at the Δ12 or Δ15 positions. For this reason, linoleate (18:2 Δ9,12) and linolenate (18:3 Δ9,12,15) must be obtained from the diet and, thus, are termed essential fatty acids. These dietary fatty acids come predominately from plant sources, since flowering plants readily desaturate the Δ12 and the Δ15 positions. Certain invertebrate animals, including some insects and nematodes, can synthesize de novo all their component fatty acids including linoleate and linolenate. The nematode *C. elegans*, for example, can synthesize de novo a broad range of polyunsaturated fatty acids including arachidonic acid and eicosapentaenoic acids, a feature not shared by either mammals or flowering plants (Spychalla et al. (1997) *Proc. Natl. Acad. Sci USA* 94(4):1142-7).

The *C. elegans* desaturase gene *fat2* has been expressed in *S. cerevisiae* and shown to be delta-12 fatty acid desaturase (Peyou-Ndi et al. (2000) *Arch. Biochem. Biophys.* 376(2):399-408). This enzyme introduces a double bond between the 12th and the 13th carbons (from the carboxylate end) and can convert the mono-unsaturated oleate (18:1 Δ9) and palmitoleate (16:1 Δ9) to the di-unsaturated linoleate (18:2 Δ9,12) and 16:2 Δ9,12 fatty acids, respectively.

The nematode delta-12 enzymes are potentially good targets for anti-nematode compounds for several reasons. Firstly, as mentioned above, mammals are thought not to have delta-12 fatty acid desaturases. In addition, the nematode enzymes appear to be phylogenetically distinct from their homologs in plants, having less than 40% pairwise sequence identity at the amino acid level and phylogenetic analyses demonstrate clustering of nematode delta-12 and ω-3 desaturases away from homologs in plants. Experiments with both transgenic *Arabidopsis* and soybeans reveal that plants can tolerate significant reductions in linoleate or linolenate, suggesting that inhibitors of delta-12 desaturases would likely not be toxic to plants (Miquel & Browse (1992) *J. Biol. Chem.* 267(3):1502-9; Singh et al. (2000) *Biochem. Society Trans.* 28: 940-942; Lee et al. (1998) *Science* 280:915-918). Thus, inhibitors of the enzyme are likely to be non-toxic to mammals.

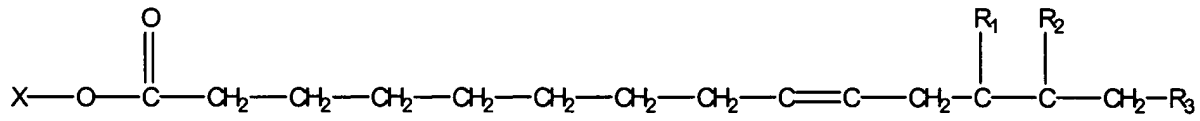
We made the surprising discovery that the parent fatty acids and methyl esters of certain fatty acid analogs (e.g., ricinoleate, vernolate) are nematicidal and have activity consistent with that of specific inhibitors of nematode delta-12 desaturases. The fatty acids and methyl esters show significantly increased anthelmintic activity compared to eighteen carbon free fatty acids and esters such as oleate, elaidate and linoleate. In contrast to short

chain fatty acids and esters such as pelargonate (pelargonic acid or methyl pelargonate), fatty acid analogs that are predicted delta-12 desaturase inhibitors show reduced phytotoxicity and can therefore be used effectively while minimizing undesirable damage to non-target organisms. Suitable nematode-inhibitory compounds include compounds having the following fatty acids in free or esterified form: ricinoleic acid (12-hydroxoctadec-cis-9-enoic acid), hydroxypalmitoleic acid (12-hydroxyhexadec-cis-9-enoic acid), ricinelaidic acid, vernolic acid ((12,13)-epoxy-octadec-cis-9-enoic acid), and 12-oxo-9(Z)-octadecenoic acid.

Polypeptides

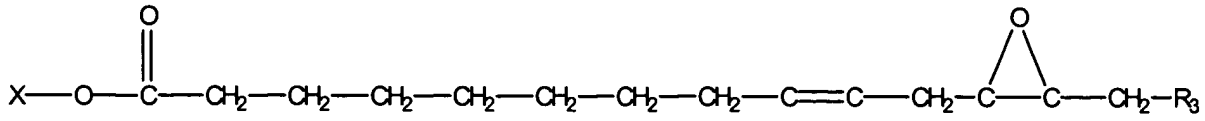
A polypeptide suitable for use in the invention is effective for catalysing the conversion of a substrate to a C16, C18, or C20 monounsaturated fatty acid product, e.g., a hydroxylated fatty acid or an epoxygenated fatty acid. The enzymatic products of hydroxylase or epoxygenase enzymes useful in the invention typically are fatty acids 16, 18, or 20 carbons in length, or analogs thereof. Such products typically have a cis (Z) or a trans (E) carbon double bond at the delta-9 position, between C9 and C10 counting from the carbonyl (carboxyl) carbon. Such products also have hydroxy or epoxy modifications at C12, C13 or both C12 and C13. A fatty acid hydroxylase or epoxygenase of this invention includes a polypeptide that demonstrates the ability to catalyze the production of ricinoleic, lesquerolic, hydroxyerucic (16-hydroxydocos-cis-13-enoic acid) or hydroxypalmitoleic (12-hydroxyhexadec-cis-9-enoic) from Coenzyme A, acyl carrier protein (ACP) or lipid-linked monoenoic fatty acid substrates under suitable conditions.

In some embodiments, the product is a C16-C20 monounsaturated oxo-fatty acid that has the following structure:



One or both of R_1 and R_2 can be hydroxyl, e.g., R_1 is hydrogen and R_2 is hydroxyl, R_1 is hydroxyl and R_2 is hydrogen, or both R_1 and R_2 are hydroxyl. Alternatively, R_1 can be keto and R_2 hydrogen, or R_1 can be hydrogen and R_2 keto. R_3 can be C2 alkyl, C4 alkyl, or C6 alkyl.

In other embodiments, the product is a C16-C20 epoxy monounsaturated fatty acid product that has the following structure:



If X is hydrogen in the structures given above, the product is a free fatty acid. However, X can also be CoA, ACP, phosphatidylcholine, or phosphatidylethanolamine. X can also be glycerol, a glyceride, methyl, or Na+. In both of the structures given above, the double bond between the 9th and 10th carbons can be *cis* or can be *trans*.

Whether a polypeptide exhibits hydroxylase activity or epoxygenase activity can be determined by testing the polypeptide e.g., in a hydroxylase assay described in US 6,310,194, or an epoxygenase assay described in US 6,329,518. A rapid and efficient method to identify suitable polypeptides is an analysis of fatty acid production in yeast that express the polypeptide to be tested. Since *Saccharomyces cerevisiae* does not produce linoleic acid (the substrate of delta-12 desaturase-like epoxygenases), linoleic acid or methyl linoleate is provided exogenously as a substrate. Any conversion of the substrate to a hydroxylated or epoxygenated product can be measured by, for example, gas chromatography-mass spectrometry (GC-MS) of total fatty acids after hydrolysis and conversion to methyl esters. A polypeptide is considered to have hydroxylase activity or epoxygenase activity when it produces an amount of hydroxy- or epoxy-fatty acid that is statistically significantly greater in *Saccharomyces cerevisiae* that express the polypeptide, relative to the amount produced in corresponding control *S. cerevisiae* that lack or do not express the polypeptide. An alternative technique for identifying suitable polypeptides is an analysis of fatty acid content in vegetative tissues of *Arabidopsis* plants, e.g., leaf tissue or root tissue.

Typically, a difference is considered statistically significant a $p \leq 0.05$ with an appropriate parametric or non-parametric statistic, e.g., Chi-square test, Student's t-test, Mann-Whitney test, or F-test. In some embodiments, a difference is statistically significant at $p < 0.01$, $p < 0.005$, or $p < 0.001$. A statistically significant difference in, for example, the level of ricinoleic acid in seeds from a transgenic *Arabidopsis* plant that expresses a hydroxylase polypeptide, compared to the level in a control *Arabidopsis* plant, indicates that

expression of the polypeptide results in an increase in the level of ricinoleic acid. The significantly increased amount of a hydroxy-fatty acid can constitute from about 0.01% to about 25% by weight of the total fatty acid content of a sample, e.g., from about 0.03% to about 20%, about 0.05% to about 20%, about 0.1% to about 10%, about 0.1% to about 5%, about 0.2% to about 3%, about 0.5% to about 5.0%, about 0.5% to about 10%, about 2.0% to about 15%, about 1.0% to about 5.0%, about 1.0% to about 10%, about 3% to about 8%, about 3% to about 10%, about 4% to about 9%, about 4% to about 13%, about 5% to about 20%, about 5% to about 15%, or about 5% to about 10%. The significantly increased amount of an epoxy-fatty acid can constitute from about 0.01% to about 35% by weight of the total fatty acid content of a sample, e.g., from about 0.03% to about 25%, about 0.05% to about 20%, about 0.1% to about 5%, about 0.2% to about 3%, about 0.5% to about 5.0%, about 0.5% to about 10%, about 2.0% to about 15%, about 1.0% to about 5.0%, about 1.0% to about 10%, about 3% to about 8%, about 3% to about 10%, about 4% to about 9%, about 4% to about 13%, about 5% to about 20%, about 5% to about 15%, or about 5% to about 10%.

In some embodiments, the polypeptide is a hydroxylase encoded by a gene isolated from *Lesquerella* or *Ricinus* plants. In other embodiments, the polypeptide is an epoxygenase encoded by a gene isolated from *Stokesia*, *Crepis* or *Vernonia* plants. Examples of these enzymes include the oleate hydroxylases from *Ricinus communis*, *Lesquerella fendleri*, *Lesquerella lindheimeri*, *Lesquerella gracilis* and linoleate epoxygenases from *Stokesia laevis*, *Crepis biennis*, *Crepis palaestina* and *Vernonia galamensis*.

In some embodiments, a polypeptide suitable for use in the invention is a fusion of two or more naturally-occurring amino acid sequences. For example, a naturally occurring oleate hydroxylase polypeptide derived from *Ricinus communis*, *Lesquerella fendleri*, *Lesquerella lindheimeri*, or *Lesquerella gracilis* can have approximately thirty amino acids at the N-terminus replaced by N-terminal amino acids from the *Arabidopsis thaliana* FAD2 gene. See, e.g., SEQ ID NOS: 19 through 23. Alternatively, a fusion polypeptide can be a naturally occurring linoleate epoxygenase derived from *Stokesia laevis* or *Crepis biennis* where amino acids at the N-terminus are replaced by N-terminal amino acids from the *Arabidopsis thaliana* FAD2 gene.

Other naturally occurring hydroxylases and epoxygenases are obtainable using the specific exemplified sequences provided herein. Furthermore, it will be apparent that one can make synthetic hydroxylases having modified amino acid sequences. Modified amino acid sequences include sequences which have been mutated, truncated, increased and the like, whether such sequences were partially or wholly synthesized.

In some embodiments, a hydroxylase or epoxygenase suitable for use in the invention has at least 60% overall amino acid sequence identity with a target polypeptide, e.g., 75%, 80%, 85%, 90%, 95%, 96%, 98%, or 99% sequence identity.

A percent identity for any subject nucleic acid or amino acid sequence (*e.g.*, any of the hydroxylase polypeptides described herein) relative to another "target" nucleic acid or amino acid sequence can be determined as follows. First, a target nucleic acid or amino acid sequence can be compared and aligned to a subject nucleic acid or amino acid sequence, using the BLAST 2 Sequences (Bl2seq) program from the stand-alone version of BLASTZ containing BLASTN and BLASTP (*e.g.*, version 2.0.14). The stand-alone version of BLASTZ can be obtained at <www.fr.com/blast> or www.ncbi.nlm.nih.gov>. Instructions explaining how to use BLASTZ, and specifically the Bl2seq program, can be found in the 'readme' file accompanying BLASTZ. The programs also are described in detail by Karlin et al. (1990) *Proc. Natl. Acad. Sci.* 87:2264; Karlin et al. (1990) *Proc. Natl. Acad. Sci.* 90:5873; and Altschul et al. (1997) *Nucl. Acids Res.* 25:3389.

Bl2seq performs a comparison between the subject sequence and a target sequence using either the BLASTN (used to compare nucleic acid sequences) or BLASTP (used to compare amino acid sequences) algorithm. Typically, the default parameters of a BLOSUM62 scoring matrix, gap existence cost of 11 and extension cost of 1, a word size of 3, an expect value of 10, a per residue cost of 1 and a lambda ratio of 0.85 are used when performing amino acid sequence alignments. The output file contains aligned regions of homology between the target sequence and the subject sequence. Once aligned, a length is determined by counting the number of consecutive nucleotides or amino acid residues (*i.e.*, excluding gaps) from the target sequence that align with sequence from the subject sequence starting with any matched position and ending with any other matched position. A matched position is any position where an identical nucleotide or amino acid residue is present in both the target and subject sequence. Gaps of one or more residues can be inserted into a target or

subject sequence to maximize sequence alignments between structurally conserved domains (*e.g.*, α-helices, β-sheets, and loops).

The percent identity over a particular length is determined by counting the number of matched positions over that particular length, dividing that number by the length and multiplying the resulting value by 100. For example, if (*i*) a 500 amino acid target sequence is compared to a subject amino acid sequence, (*ii*) the Bl2seq program presents 200 amino acids from the target sequence aligned with a region of the subject sequence where the first and last amino acids of that 200 amino acid region are matches, and (*iii*) the number of matches over those 200 aligned amino acids is 180, then the 500 amino acid target sequence contains a length of 200 and a sequence identity over that length of 90% (*i.e.*, 180 ÷ 200 x 100 = 90). In some embodiments, the amino acid sequence of a polypeptide suitable for use in the invention has 40% sequence identity to the amino acid sequence of SEQ ID NOS: 13, 14, 15, 16, 17, 18 or 36. In other embodiments, the amino acid sequence of a polypeptide suitable for use in the invention has greater than 40% sequence identity (e.g., > 40%, > 50%, > 60%, > 70%, > 80%, > 90%, or >95%) to the amino acid sequence of SEQ ID NOS: 13, 14, 15, 16, 17, 18 or 36.

It will be appreciated that a nucleic acid or amino acid target sequence that aligns with a subject sequence can result in many different lengths with each length having its own percent identity. It is noted that the percent identity value can be rounded to the nearest tenth. For example, 78.11, 78.12, 78.13, and 78.14 is rounded down to 78.1, while 78.15, 78.16, 78.17, 78.18, and 78.19 is rounded up to 78.2. It is also noted that the length value will always be an integer.

The identification of conserved regions in a template, or subject, polypeptide can facilitate homologous polypeptide sequence analysis. Conserved regions can be identified by locating a region within the primary amino acid sequence of a template polypeptide that is a repeated sequence, forms some secondary structure (e.g., helices and beta sheets), establishes positively or negatively charged domains, or represents a protein motif or domain. See, e.g., the Pfam web site describing consensus sequences for a variety of protein motifs and domains at http://www.sanger.ac.uk/Pfam/ and http://genome.wustl.edu/Pfam/. A description of the information included at the Pfam database is described in Sonnhammer et al. (1998) *Nucl. Acids Res.* 26: 320-322; Sonnhammer et al. (1997) *Proteins* 28:405-420; and

Bateman et al. (1999) *Nucl. Acids Res.* 27:260-262. From the Pfam database, consensus sequences of protein motifs and domains can be aligned with the template polypeptide sequence to determine conserved region(s).

Conserved regions also can be determined by aligning sequences of the same or related polypeptides from closely related plant species. Closely related plant species preferably are from the same family. Alternatively, alignments are performed using sequences from plant species that are all monocots or are all dicots. In some embodiments, alignment of sequences from two different plant species is adequate. For example, sequences from canola and *Arabidopsis* can be used to identify one or more conserved regions.

Typically, polypeptides that exhibit at least about 35% amino acid sequence identity are useful to identify conserved regions. Conserved regions of related proteins sometimes exhibit at least 40% amino acid sequence identity (e.g., at least 50%, at least 60%; or at least 70%, at least 80%, or at least 90% amino acid sequence identity). In some embodiments, a conserved region of target and template polypeptides exhibit at least 92, 94, 96, 98, or 99% amino acid sequence identity. Amino acid sequence identity can be deduced from amino acid or nucleotide sequence.

A polypeptide useful in the invention optionally can possess additional amino acid residues at the amino-terminus or the carboxy-terminus. For example, 6x His-tag or FLAG™ residues can be linked to a polypeptide at the amino-terminus. See, *e.g.*, U.S. Patent Nos. 4,851,341 and 5,001,912. As another example, a reporter polypeptide such as green fluorescent protein (GFP) can be fused to the carboxy-terminus of the polypeptide. See, for example, U.S. Patent No. 5,491,084.

Nucleic Acids

Among the nucleic acids suitable for the invention are those that encode a polypeptide described herein. Typically, such a nucleic acid is incorporated into a DNA construct suitable for introduction into a plant and integration into a plant genome. A DNA construct comprising a nucleic acid encoding a hydroxylase or epoxygenase polypeptide is operably linked to one or more regulatory elements that confer expression in vegetative tissues of a plant. Typically, a DNA construct includes a 5'-regulatory element and a 3'-regulatory element for expression in transformed plants. In some embodiments, such

constructs are chimeric, i.e., the coding sequence and one or more of the regulatory sequences are from different sources. For example, a polypeptide coding sequence can be a *Ricinus communis* hydroxylase and a 5'-regulatory element can be a potato S27a promoter. However, non-chimeric DNA constructs also can be used. DNA constructs can also include cloning vector nucleic acids. Cloning vectors suitable for use in the present invention are commercially available and are used routinely by those of ordinary skill in the art.

Regulatory elements typically do not themselves code for a gene product. Instead, regulatory elements affect expression of the coding sequence, i.e., transcription of the coding sequence, and processing and translation of the resulting mRNA. Examples of regulatory elements suitable for use in a DNA construct include promoter sequences, enhancer sequences, response elements or inducible elements that modulate expression of a nucleic acid sequence. As used herein, "operably linked" refers to positioning of a regulatory element in a construct relative to a nucleic acid coding sequence in such a way as to permit or facilitate expression of the encoded polypeptide. The choice of element(s) that are included in a construct depends upon several factors, including, but not limited to, replication efficiency, selectability, inducibility, desired expression level, and cell or tissue specificity.

Suitable regulatory elements include promoters that initiate transcription only, or predominantly, in certain cell types. For example, promoters specific to vegetative tissues such as ground meristem, vascular bundle, cambium, phloem, cortex, shoot apical meristem, lateral shoot meristem, root apical meristem, lateral root meristem, leaf primordium, leaf mesophyll, or leaf epidermis can be suitable regulatory elements. A cell type or tissue-specific promoter can drive expression of operably linked sequences in tissues other than vegetative tissue. Thus, as used herein a cell type or tissue-specific promoter is one that drives expression preferentially in the target tissue, but can also lead to some expression in other cell types or tissues as well. Methods for identifying and characterizing promoter regions in plant genomic DNA include, for example, those described in the following references: Jordano et al. (1989) *Plant Cell*, 1:855-866; Bustos et al. (1989) *Plant Cell*, 1:839-854; Green et al. (1988) *EMBO J.* 7:4035-4044; Meier et al. (1991) *Plant Cell*, 3:309-316; and Zhang et al. (1996) *Plant Physio.* 110:1069-1079.

Other suitable regulatory elements can be found in 5'-untranslated regions (5'-UTR) and 3'-untranslated regions (3'-UTR). The terms 5'-UTR and 3'-UTR refer to nucleic acids

that are positioned 5' and 3' to a coding sequence, respectively, in a DNA construct and that can be found in mRNA 5' to the initiation codon and 3' to the stop codon, respectively. A 5'-UTR and a 3'-UTR can include elements that affect transcription of the coding sequence, as well as elements that affect processing of mRNA and translation of the coding sequence.

Regulatory elements suitable for use in plants include nopaline and mannopine synthase regulatory elements, cauliflower mosaic virus 35S promoters, *Arabidopsis* root periphery IRT2 promoter, *Solanum tuberosum* (potato) ribosomal S27a Ubi3 promoter, rice Actin I gene promoter and Ubiquitin I gene promoter from maize (McElroy et al. (1995) *Mol. Breed.* 1:27-37). Inducible nematode responsive promoters of interest include the tobacco tobRB7 (Yamamoto et al. (1991) *Plant Cell,* 3(4):371-382), sunflower Sun-RB7 (Sarda et al. (1999) *Plant Mol Biol.* 40(1):179-191) and potato potRB7 (Heinrich et al. (1996) *Plant Physiol.* 112(2):861-864) promoters. Other exemplary promoter-5'-UTR constructs which can be used in applications requiring root expression are listed in Table 8.

For embodiments where expression of a polypeptide is desired in vegetative plant tissues such as leaves or roots, the use of all or part of the 5' upstream non-coding regions (5'-UTR) and 3' downstream non-coding regions (3'-UTR) of a *Arabidopsis* FAD2 or FAD3 gene are contemplated. Also suitable is the construction of chimeric hydroxylases and epoxygenases by swapping approximately the first 30 amino acids from a desaturase such as the FAD2 or FAD3 desaturases for the equivalent N-terminal region of the hydroxylase or epoxygenase as in SEQ ID NOS: 7 to 12 and 19 to 24. Particularly desirable are the use of chimeric desaturase-like epoxygenases or hydroxylases with non-seed specific UTRs.

Regulatory elements such as transcript termination regions may be provided in DNA constructs. If the coding sequence and the transcript termination region in a DNA construct are derived from different naturally occurring sources, the transcript termination region typically contains at least about 0.5 kb, preferably about 1-3 kb of sequence 3' to the structural gene from which the termination region is derived.

DNA constructs also can contain sequences encoding other polypeptides. Such polypeptides can, for example, facilitate the introduction or maintenance of the nucleic acid construct in a host organism. Potential host cells include both prokaryotic and eukaryotic cells. A host cell may be unicellular or found in a multicellular differentiated or undifferentiated organism depending upon the intended use. Depending upon the host,

regulatory elements can include elements from viral, plasmid or chromosomal genes, or the like. For expression in prokaryotic or eukaryotic microorganisms, particularly unicellular hosts, a wide variety of constitutive or inducible promoters may be employed. Expression in a microorganism can provide a ready source of a desired polypeptide. Among transcriptional initiation regions which have been described are regions from bacterial and yeast hosts, such as *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, including genes such as beta-galactosidase, T7 polymerase, tryptophan E and the like.

DNA constructs can also include sequences encoding other polypeptides that can affect the expression, activity, biochemical activity or physiological activity of a hydroxylase or epoxygenase polypeptide. For example, a DNA construct can include a nucleic acid encoding a PDAT, DAGAT, lipase, FAD2 or FAD3 polypeptide, operably linked to at least one regulatory element that confers expression in vegetative tissues of a plant. In some embodiments, a DNA construct includes a nucleic acid that encodes a PDAT polypeptide and a nucleic acid that encodes a FAD2 polypeptide. Alternatively, such other polypeptide coding sequences can be provided on a separate DNA construct(s).

Suitable phospholipid:diacylglycerol acyltransferase (PDAT) polypeptides and diacylglycerol acyltransferase (DAGAT) polypeptides include *A. thaliana* DAGAT or *C. elegans* DAGAT. Coding sequences for suitable PDAT and DAGAT polypeptides include GenBank® Accession Nos. AAF19262, AAF19345, AAF82410 and P40345.

DAGAT and PDAT enzymes are important determinants of both the amounts (Bouvier-Nave et al. (2000) *Biochem. Soc. Trans.* 28(6):692-695; Jako et al. (2001) 126(2):861-874) and types (Banas et al. (2000) *Biochem. Soc. Trans.* 28(6):703-705; Dahlqvist et al. (2000) *Proc. Natl. Acad. Sci USA,* 97(12):6487-6492) of fatty acids found in the triacylglycerol (TAG) fraction. Furthermore, the triacylglycerol (TAG) fraction is the predominant repository of novel fatty acids like ricinoleic acid and vernolic acid in seeds and it is thought that this minimizes the disruptive effects of these unusual fatty acids on plant cell membranes (Millar et al. (2000) *Trends Plant Sci.* 5(3):95-101). In most plants, roots, leaves, and other non-seed tissues are not usually sites of major triacylglycerol accumulation. It is therefore likely that in non-seed tissues the activity of key enzymes in the TAG synthesis pathway such as PDATs and DAGATs are suboptimal for the contemplated application and can be improved by overexpression of these enzymes which can result in significant

enhancement of fatty acid accumulation in the TAG fraction (Bouvier-Nave et al. (2000) *Eur. J. Biochem.* 267(1):85-96).

A DNA construct that encodes one or more desaturases includes constructs that encode delta-12 fatty acid desaturases or delta-15 fatty acid desaturases. For example, an *Arabidopsis thaliana* FAD2 or an *Arabidopsis thaliana* FAD3 polypeptide can be operably linked to a suitable promoter that confers expression in non-seed tissues such as roots and/or leaves. The expression of a delta-12 desaturase and an epoxygenases can be useful, since linoleic acid, the product of the desaturase, is the substrate converted to vernolic acid by the epoxygenase.

Nucleic acids describe herein can be used to identify homologous plant hydroxylase or epoxygenase coding sequences and the resulting sequences may provide further plant hydroxylases or epoxygenases. In particular, PCR may be a useful technique to obtain related nucleic acids from sequence data provided herein. One skilled in the art will be able to design oligonucleotide probes based upon sequence comparisons or regions of typically highly conserved sequence. Of special interest are polymerase chain reaction primers based on the conserved regions of amino acid sequence between the hydroxylases and epoxygenases in Figure 2 (SEQ ID NOS: 13 to 18 and SEQ ID NO 36). Details relating to the design and methods for a PCR reaction using these probes are described more fully in the examples. If nucleic acid probes are used, they can be shorter than the entire coding sequence. Oligonucleotides may be used, for example, are 10, 15, 20, or 25 nucleotides or more in length.

Hydroxylated fatty acids are found in large quantities in some natural plant species which suggests several possibilities for plant enzyme sources. For example, hydroxy fatty acids related to ricinoleate occur in major amounts in seed oils from various *Lesquerella* species. Of particular interest, lesquerolic acid is a 20-carbon homolog of ricinoleate with two additional carbons at the carboxyl end of the chain. Other natural plant sources of hydroxylated fatty acids include seeds of the *Linum* genus, seeds of Wrightia species, *Lycopodium* species, *Strophanthus* species, *Convolvulaces* species, Calendula species and many others (van de Loo et al. (1993). For example, *Lesquerella densipila* contains a diunsaturated 18 carbon fatty acid with a hydroxyl group (van de Loo et al. (1993) *Lipid Metabolism in Plants* CRC Press, Boca Raton, p. 99-126) that is thought to be produced by

an enzyme that is closely related to the castor and *Lesquerella fendleri* hydroxylases. Similarly, epoxygenated fatty acids are found in a variety of plants including *Vernonia* genus, *Crepis* genus, *Euphorbia* genus and *Stokesia laevis*.

In addition, nucleic acids encoding a polypeptide modified from a naturally occurring sequence can be made by mutagenesis. A delta-12 desaturase can for example be converted to an oleate hydroxylase by targeted mutagenesis (Broun et al. (1998) *Science*, 282(5392):1315-1317; Broadwater et al. (2002) *J Biol Chem.* 277(18):15613-15620.). Similar changes in coding sequences such as delta-15 (omega-3) desaturases can be carried out to produce novel hydroxylases. As is well known in the art, once a cDNA clone encoding a plant hydroxylase or epoxygenase is obtained, it may be used to obtain its corresponding genomic nucleic acid. Thus, one skilled in the art will recognize that antibody preparations, nucleic acid probes and the like may be prepared and used to screen and recover homologous or related hydroxylases and epoxygenases from a variety of sources.

Typically, a nucleic acid of the invention has 70% or greater sequence identity, e.g., 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% or greater sequence identity to a target nucleic acid. Sequence identity is determined as described herein. In some embodiments, nucleic acids are from 20 to 30 nucleotides, or 20 to 50 nucleotides, or 25 to 100 nucleotides, or 500 to 1500 nucleotides, or 900 to 2,000 nucleotides in length. Specific embodiments of nucleic acids include nucleotide sequences set forth in the sequence listings. It is noted that the degeneracy of the genetic code permits codon modification without a corresponding modification of the amino acid sequence. Thus, codons in a nucleic acid can be modified if desired, which may optimize expression of a polypeptide. For example, codons with 8% or lower percentage occurrence in a selected plant species genome can be replaced with a more frequently occurring codon, e.g., the most frequent or second most frequent codon for that particular amino acid. As another alternative, one member of a contiguous pair of codons can be modified if both codons have an occurrence of 12% or lower in known sequences of the genome of a selected plant species. Data relating to codon usage database can be found, for example, at <http://www.kazusa.or.jp/codon/>. Codons can also be changed to remove ATTTA (i.e., AUUUA) elements which may contribute to mRNA instability, and codons may be changed to ablate potential polyadenylation sites. Codons can also be modified to break up runs of five or greater contiguous nucleotides of A, G, C or T (e.g., TTTTTT).

Codons can also be modified to reduce the likelihood of aberrant splicing. Splicing potential can be assessed and donor (GT) or acceptor (AG) splice sites ablated in order to diminish splicing potential, using predictive algorithms such as algorithms at <http://www.cbs.dtu.dk/services/NetPGene>. In addition, codons near the N-terminus of the polypeptide can be changed to codons preferred by a selected plant species, e.g, soybean (*Glycine max*). It will be appreciated that one or more codon modifications, including but not limited to the modifications discussed above can be made to a nucleic acid coding sequence. Examples of sequences that have one or more codon modification(s) to improve plant expression and have slight changes to the amino acid sequences relative to the wild-type sequence include SEQ ID NOS: 28 through 33.

A nucleic acid encoding a polypeptide can have a genomic coding sequence, a cDNA coding sequence, or an mRNA coding sequence. A cDNA coding sequence may or may not have pre-processing sequences, such as transit or signal peptide sequences. Transit or signal peptide sequences facilitate the delivery of the protein to a given organelle and are frequently cleaved from the polypeptide upon entry into the organelle, releasing the "mature" sequence. The use of the precursor DNA sequence can be useful in plant cell expression cassettes.

Transgenic Plants

According to another aspect of the invention, transgenic plants are provided. Such plants typically express the polypeptide coding sequence of a DNA construct described herein, resulting in an increase in the amount of a hydroxylated or epoxygenated fatty acid in vegetative plant tissues. A plant species or cultivar may be transformed with a DNA construct that encodes a polypeptide from a different plant species or cultivar (e.g., soybean transformed with a gene encoding a castor enzyme). Alternatively, a plant species or cultivar may be transformed with a DNA construct that encodes a polypeptide from the same plant species or cultivar.

Accordingly, a method according to the invention comprises introducing a DNA construct as described herein into a plant. Techniques for introducing exogenous nucleic acids into monocotyledonous and dicotyledonous plants are known in the art, and include, without limitation, *Agrobacterium*-mediated transformation, liposome fusion, microinjection, viral vector-mediated transformation, infiltration, imbibition, electroporation and particle gun

transformation, e.g., U.S. Pat. Nos 5,204,253 and 6,013,863. If a cell or tissue culture is used as the recipient tissue for transformation, plants can be regenerated from transformed cultures by techniques known to those skilled in the art. Any method that provides for transformation may be employed.

Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the Agrobacterium host for homologous recombination with the Ti- or Ri-plasmid present in the *Agrobacterium* host. The Ti- or Ri-plasmid containing the T-DNA for recombination may be armed (capable of causing gall formation) or disarmed (incapable of causing gall), the latter being permissible, so long as the *vir* genes are present in the transformed Agrobacterium host. The armed plasmid can give a mixture of normal plant cells and gall.

In some instances where *Agrobacterium* is used as the vehicle for transforming plant cells, the DNA construct, bordered by the T-DNA border(s), will be inserted into a broad host spectrum vector, there being broad host spectrum vectors described in the literature. Commonly used is pRK2 or derivatives thereof. Included with the expression construct and the T-DNA will be one or more markers, which allow for selection of transformed *Agrobacterium* and transformed plant cells. A number of markers have been developed for use with plant cells, such as resistance to kanamycin, the aminoglycoside G418, hygromycin, or the like.

A number of genes that confer herbicide resistance can be used as markers. Genes conferring resistance to a herbicide that inhibits the growing point or meristem can be suitable. Exemplary genes in this category code for mutant ALS and AHAS enzymes as described, for example, in U.S. 5,767,366 and 5,928,937. U.S. Pat. Nos. 4,761,373 and 5,013,659 are directed to plants resistant to various imidazolinone or sulfonamide herbicides. U.S. Pat. No. 4,975,374 relates to plant cells and plants containing a gene encoding a mutant glutamine synthetase (GS) resistant to inhibition by herbicides that are known to inhibit GS, *e.g.* phosphinothricin and methionine sulfoximine. U.S. Pat. No. 5,162,602 discloses plants resistant to inhibition by cyclohexanedione and aryloxyphenoxypropanoic acid herbicides. The resistance is conferred by an altered acetyl coenzyme A carboxylase(ACCase). Genes for resistance to glyphosate (sold under the trade name Roundup®) are also suitable. See, for example, U.S. Pat. No. 4,940,835 and U.S. Pat. No. 4,769,061. U.S. Pat. No. 5,554,798

discloses transgenic glyphosate resistant maize plants, which resistance is conferred by an altered 5-enolpyruvyl-3-phosphoshikimate (EPSP) synthase gene. Genes for resistance to phosphono compounds such as glufosinate ammonium or phosphinothricin, and pyridinoxy or phenoxy propionic acids and cyclohexones are also suitable. See European application No. 0 242 246. Other suitable herbicides include those that inhibit photosynthesis, such as a triazine and a benzonitrile (nitrilase). See U.S. Pat. No. 4,810,648. Other suitable herbicides include 2,2-dichloropropionic acid, sethoxydim, haloxyfop, imidazolinone herbicides, sulfonylurea herbicides, triazolopyrimidine herbicides, s-triazine herbicides and bromoxynil. Also suitable are herbicides that confer resistance to a protox enzyme. See, *e.g.*, U.S. Patent Application No. 20010016956, and U.S. Pat. No. 6,084,155. The particular marker employed is not essential to this invention, one or another marker being suitable depending on the particular host and the manner of construction.

Transgenic plants typically contain a DNA construct integrated into their genome and typically exhibit Mendelian inheritance patterns. Transgenic plants can be entered into a breeding program, e.g., to introduce a nucleic acid encoding a polypeptide into other lines, to transfer the nucleic acid to other species or for further selection of other desirable traits. Alternatively, transgenic plants can be propagated vegetatively for those species amenable to such techniques. Progeny includes descendants of a particular plant or plant line. Progeny of an instant plant include seeds formed on F_1, F_2, F_3, and subsequent generation plants, or seeds formed on BC_1, BC_2, BC_3, and subsequent generation plants. Seeds produced by a transgenic plant can be grown and then selfed (or outcrossed and selfed) to obtain seeds homozygous for the nucleic acid encoding a novel polypeptide.

Plants which may be employed in practicing the present invention include, but are not limited to, tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), soybean (*glycine max*), peanuts (*Arachis hypogaea*), cotton (*Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (Manihot esculenta), coffee (*Cofea spp.*), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus spp.*), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (Musa spp.), avocado (*Persea americana*), fig (Ficus casica), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), corn (*Zea mays*), wheat, oats, rye, barley,

rice, vegetables, ornamentals, and conifers. Vegetables include tomatoes (*Lycopersicon esculentum*), lettuce (e.g., *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus spp.*) and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals include azalea (*Rhododendron spp.*), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa spp.*), tulips (*Tulipa spp.*), daffodils (*Narcissus spp.*), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherima*), and chrysanthemum. Conifers which may be employed in practicing the present invention include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*). Suitable grasses include Kentucky bluegrass (*Poa pratensis*) and creeping bentgrass (*Agrostris palustris*).

It is understood that hydroxylated or epoxygenated fatty acids produced by a polypeptide of the invention in planta may be subject to further enzymatic modification by other enzymes which are normally present in a plant or are introduced by genetic engineering methods into a plant. For example, lesquerolic acid, which is present in many *Lesquerella* species, is thought to be produced by elongation of ricinoleic acid (Moon et al. (2001) *Plant Physiol.* 127(4):1635-1643). Thus, the presence of a *Ricinus communis* hydroxylase construct in a transgenic plant may be sufficient produce lesquerolic acid in the same plant, via production of ricinoleic acid by the hydroxylase polypeptide and elongation of ricinoleic acid by an endogenous polypeptide.

Nematode Resistance

Transgenic plants may be tested for hydroxy- and epoxy-fatty acid production in non-seed tissues. Such plants may also be tested for nematicidal activity. Similar tests for hydroxylated and epoxygenated fatty acid production and nematicidal activity may be carried out on hairy root cultures formed by transformation with *A. rhizogenes*. Accordingly, the

invention features a method of screening a transgenic plant for anthelmintic activity, comprising contacting the plant with a nematode under conditions effective to determine whether or not the plant has anthelmintic activity. The transgenic plant has a nucleic acid encoding a hydroxylase or epoxygenase polypeptide described herein. Suitable conditions for determining anthelmintic activity are described herein. The method can also be carried out with plant tissue, e.g., root tissue, leaf tissue or stem tissue from a transgenic plant.

In another aspect, the invention features a method for making a plant having anthelmintic activity. As discussed herein, techniques for introducing exogenous nucleic acids into monocotyledonous and dicotyledonous plants are known in the art. In some embodiments, for example, a method of making a plant having anthelmintic activity comprises (1) transforming regenerable cells of a plant species with a DNA construct described herein; and (2) regenerating one or more transgenic plants from the cells. The resulting transgenic plant can have a statistically significant increase in the amount of hydroxylated or epoxygenated fatty acid in non-seed tissues compared to a corresponding untransformed counterpart. The increased level of hydroxy- or epoxy-fatty acids can result in plants that have anthelmintic activity. Nematodes that parasitize plant roots, stems, bulbs, or leaves can be controlled using the method of this invention.

As used herein, a fatty acid compound has anthelmintic activity when, tested in planta, the compound has a statistically significant increase in nematode-killing activity, a statistically significant reduction in nematode fertility, a statistically significant increase in nematode sterility, a statistically significant reduction in the ability of a nematode to infect or reproduce in its host, a statistically significant reduction in nematode growth or development, relative to a control treatment in the absence of the compound. A compound having anthelmintic activity can, for example, reduce the survival time of adult nematodes relative to unexposed similarly staged adults, e.g., by about 20%, 40%, 60%, 80%, or more. In some embodiments, a compound having anthelmintic activity may also cause the nematodes to cease replicating, regenerating, and/or producing viable progeny, e.g., by about 20%, 40%, 60%, 80%, or more, compared to a control treatment in the absence of the compound.

A compound having anthelmintic activity can result in a statistically significant increase in nematode repellant properties relative to a control treatment in the absence of the compound. In the assay, the compound is combined with nematodes, e.g., in a well of

microtiter dish, in liquid or solid media or in the soil containing the compound. Staged adult nematodes are placed on the media. The time of survival, viability of offspring, and/or the movement of the nematodes are measured.

Exemplary plants-parasitic nematodes from which plants may be protected by the present invention, and their corresponding plants, are as follows: alfalfa: *Ditylenchus dipsaci, Meloidogyne hapla, Meloidogyne incognita, Meloidogyne javanica, Pratylenchus spp., Paratylenchus spp., Xiphinema spp.*; banana: *Radopholus similis, Helicotylenchus multicinctus, Meloidogyne incognita, M. arenaria, M. javanica, Pratylenchus coffeae, Rotylenchulus reniformis*; beans and peas: *Meloidogyne spp., Heterodera spp., Belonolaimus spp., Helicotylenchus spp., Rotylenchulus reniformis, Paratrichodorus anemones, Trichodorus spp.*; cassava: *Rotylenchulus reniformis, Meloidogyne spp.*; cereals: *Anguina tritici* (Emmer, rye, spelt wheat), *Bidera avenae* (oat, wheat), *Ditylenchus dipsaci* (rye, oat), *Subanguina radicicola* (oat, barley, wheat, rye), *Meloidogyne naasi* (barley, wheat, rye), *Pratylenchus spp.* (oat, wheat, barley, rye), *Paratylenchus spp.* (wheat), *Tylenchorhynchus spp.* (wheat, oat); chickpea: *Heterodera cajani, Rotylenchulus reniformis, Hoplolaimus seinhorsti, Meloidogyne spp., Pratylenchus spp.*; citrus: *Tylenchulus semipenetrans, Radopholus similis, Radopholus citrophilus* (Florida only), *Hemicycliophora arenaria, Pratylenchus spp., Meloidogyne spp., Bolonolaimus longicaudatus* (Florida only), *Trichodorus, Paratrichodorus, Xiphinema spp.*; clover: *Meloidogyne spp., Heterodera trifolii*; coconut: *Rhadinaphelenchus cocophilus*; coffee: *Meloidogyne incognita* (most important in Brazil), *Meloidogyne exigua* (widespread), *Pratylenchus coffeae, Pratylenchus brachyurus, Radopholus similis, Rotylenchulus reniformis, Helicotylenchus spp.*; corn: *Pratylenchus spp., Paratrichodorus minor, Longidorus spp., Hoplolaimus columbus*; cotton: *Meloidogyne incognita, Belonolaimus longicaudatus, Rotylenchulus reniformis, Hoplolaimus galeatus, Pratylenchus spp., Tylenchorhynchus spp.,Paratrichodorus minor*; grapes: *Xiphinema spp., Pratylenchus vulnus, Meloidogyne spp., Tylenchulus semipenetrans, Rotylenchulus reniformis*; grasses: *Pratylenchus spp., Longidorus spp., Paratrichodorus christiei, Xiphinema spp., Ditylenchus spp.*; peanut: *Pratylenchus spp., Meloidogyne hapla., Meloidogyne arenaria, Criconemella spp., Belonolaimus longicaudatus* (in Eastern United States); pigeon pea: *Heterodera cajani, Rotylenchulus reniformis, Hoplolaimus seinhorsti, Meloidogyne spp., Pratylenchus spp.*; pineapple: *Paratrichodorus christiei, Criconemella*

spp., Meloidogyne spp., Rotylenchulus reniformis, Helicotylenchus spp., Pratylenchus spp., Paratylenchus spp.; potato: *Globodera rostochiensis, Globodera pallida, Meloidogyne spp., Pratylenchus spp., Trichodorus primitivus, Ditylenchus spp., Paratrichodorus spp., Nacoabbus aberrans*; rice: *Aphelenchiodes besseyi, Ditylenchus angustus, Hirchmanniella spp., Heterodera oryzae, Meloidogyne spp.*; small fruits: *Meloidogyne spp.; Pratylenchus spp., Xiphinema spp., Longidorus spp., Paratrichodorus christiei, Aphelenchoides spp.* (strawberry); soybean: *Heterodera glycines, Meloidogyne incognita, Meloidogyne javanica, Belonolaimus spp., Hoplolaimus columbus;* sugar beet: *Heterodera schachtii, Ditylenchus dipsaci, Meloidogyne spp., Nacobbus aberrans, Trichodorus spp., Longidorus spp., Paratrichodorus spp.*; sugar cane: *Meloidogyne spp., Pratylenchus spp., Radopholus spp., Heterodera spp., Hoplolaimus spp., Helicotylenchus spp., Scutellonema spp., Belonolaimus spp., Tylenchorhynchus spp., Xiphinema spp., Longidorus spp., Paratrichodorus spp.*; tea: *Meloidogyne spp., Pratylenchus spp., Radopholus similis, Hemicriconemoides kanayaensis, Helicotylenchus spp., Paratylenchus curvitatus*; tobacco: *Meloidogyne spp., Pratylenchus spp., Tylenchorhynchus claytoni, Globodera tabacum, Trichodorus spp., Xiphinema americanum, Ditylenchus dipsaci* (Europe only), *Paratrichodorus spp.*; tomato: *Pratylenchus spp., Meloidogyne spp.*; tree fruits: *Pratylenchus spp.* (apple, pear, stone fruits), *Paratylenchus spp.* (apple, pear), *Xiphinema spp.* (pear, cherry, peach), *Cacopaurus pestis* (walnut), *Meloidogyne spp.* (stone fruits, apple, etc.), *Longidorus spp.* (cherry), *Criconemella spp.* (peach), and *Tylenchulus spp.* (olive).

Transgenic plants described herein can provide an effective, environmentally safe means of inhibiting nematode metabolism, growth, viability, fecundity, development, infectivity and/or the nematode life-cycle. The plants may be used alone or in combination with chemical nematicides or as part of an integrated pest management strategy. Transgenic plants can afford season-long nematode control and thereby provide labor savings, by reducing the need for and frequency of chemical control.

Described below are experiments demonstrating that delta-12 fatty acid desaturase activity is essential for nematode viability. Also described are certain nematicidal fatty acids and analogs, including nematicidal fatty acids and esters that have activity consistent with that of delta-12 fatty acid desaturase inhibitors. The cloning, modification, introduction into plants and expression in non-seed tissues (e.g., roots) of DNA sequences encoding enzymes

that produce these fatty acids is also described, as are tests of regenerated plant cells, roots and plants. The following examples are to be construed as merely illustrative, and not limiting in any way whatsoever.

Example 1

RNA Mediated Interference (RNAi)

A double stranded RNA (dsRNA) molecule can be used to inactivate a delta-12 fatty acid desaturase (delta-12 *fat2*) gene in a cell by a process known as RNA mediated-interference (Fire et al. (1998) *Nature* 391:806-811, and Gönczy et al. (2000) *Nature* 408:331-336). The dsRNA molecule can have the nucleotide sequence of a delta-12 *fat2* nucleic acid (preferably exonic) or a fragment thereof. The dsRNA molecule can be delivered to nematodes via direct injection, or by soaking nematodes in aqueous solution containing concentrated dsRNA, or by raising bacteriovorous nematodes on *E. coli* genetically engineered to produce the dsRNA molecule.

RNAi by injection: To examine the effect of inhibiting delta-12 *fat2* activity, a dsRNA corresponding to the *C. elegans* delta-12 *fat2* gene was injected into the nematode, basically as described in Mello et al. (1991) *EMBO J.* 10:3959-3970. Briefly, a plasmid was constructed that contains a portion of the *C. elegans* delta-12 *fat2* sequence, specifically a fragment 651 nucleotides long, containing the entire first exon and terminating just before the conserved intron splice junction between the first exon and first intron. This construct encodes approximately the first 217 amino acids of the *C. elegans* delta-12 *fat2* gene. Primers were used to specifically amplify this sequence as a linear dsDNA. Single-stranded RNAs were transcribed from these fragments using T7 RNA polymerase and SP6 RNA polymerase (the RNAs correspond to the sense and antisense RNA strands). RNA was precipitated and resuspended in RNAse free water. For annealing of ssRNAs to form dsRNAs, ssRNAs were combined, heated to 95 °C for two minutes then allowed to cool from 70 °C to room temperature over 1.5-2.5 hours.

DsRNA was injected into the body cavity of 15-20 young adult *C. elegans* hermaphrodites. Worms were immobilized on an agarose pad and typically injected at a concentration of 1 mg/mL. Injections were performed with visual observation using a Zeiss Axiovert compound microscope equipped with 10X and 40X DIC objectives, for example.

Needles for microinjection were prepared using a Narishige needle puller, stage micromanipulator (Leitz) and an N2-powered injector (Narishige) set at 10-20 p.s.i. After injection, 200 μl of recovery buffer (0.1% salmon sperm DNA, 4% glucose, 2.4 mM KCl, 66 mM NaCl, 3 mM CaCl2, 3 mM HEPES, pH 7.2) were added to the agarose pad and the worms were allowed to recover on the agarose pad for 0.5-4 hours. After recovery, the worms were transferred to NGM agar plates seeded with a lawn of *E. coli* strain OP50 as a food source. The following day and for 3 successive days thereafter, 7 individual healthy injected worms were transferred to new NGM plates seeded with OP50. The number of eggs laid per worm per day and the number of those eggs that hatched and reached fertile adulthood were determined. As a control, Green Fluorescent Protein (GFP) dsRNA was produced and injected using similar methods. GFP is a commonly used reporter gene originally isolated from jellyfish and is widely used in both prokaryotic and eukaryotic systems. The GFP gene is not present in the wild-type *C. elegans* genome and, therefore, GFP dsRNA does not trigger an RNAi phenotype in wild-type *C. elegans*. The *C. elegans* delta-12 *fat2* RNAi injection phenotype presented as a strongly reduced F1 hatch-rate, with the few surviving individuals arrested in an early larval stage.

RNAi by feeding: *C. elegans* can be grown on lawns of *E. coli* genetically engineered to produce double stranded RNA (dsRNA) designed to inhibit delta-12 *fat2* expression. Briefly, *E. coli* were transformed with a genomic fragment of a portion of the *C. elegans fat2* gene sequence, specifically a fragment 651 nucleotides long, containing the entire first exon and terminating just before the conserved intron splice junction between the first exon and first intron. This construct encodes approximately the first 217 amino acids of the *C. elegans* delta-12 *fat2* gene. The 651 nucleotide genomic fragment was cloned into an *E. coli* expression vector between opposing T7 polymerase promoters. The clone was then transformed into a strain of *E. coli* that carries an IPTG-inducible T7 polymerase. As a control, *E. coli* was transformed with a gene encoding the Green Fluorescent Protein (GFP). Feeding RNAi was initiated from *C. elegans* eggs or from *C. elegans* L4s. When feeding RNAi was started from *C. elegans* eggs at 23 °C on NGM plates containing IPTG and *E. coli* expressing the *C. elegans* delta-12 *fat2* or GFP dsRNA, the *C. elegans* delta-12 *fat2* RNAi feeding phenotype presented as partially sterile F1 individuals and dead F2 embryos. When feeding RNAi was started from *C. elegans* L4 larvae at 23 °C on NGM plates containing

IPTG and *E. coli* expressing the *C. elegans* DELTA-12 *fat2* or GFP dsRNA, the *C. elegans* RNAi feeding phenotype presented as partially sterile P0 individuals (i.e., the individuals exposed initially) with developmentally arrested, sterile F1 nematodes. The sequence of the *fat2* gene is of sufficiently high complexity (i.e., unique) such that the RNAi is not likely to represent cross reactivity with other genes.

C. elegans cultures grown in the presence of *E. coli* expressing dsRNA and those injected with dsRNA from the delta-12 *fat2* gene were strongly impaired indicating that the fatty acid desaturase-like gene provides an essential function in nematodes and that dsRNA from the fatty acid desaturase-like gene is lethal when ingested by or injected into *C. elegans*.

Example 2

Rescue of *C. elegans* Delta-12 *fat2* RNAi Feeding Phenotype by Linoleic Acid Methyl Ester

The *C. elegans* delta-12 fatty acid desaturase (FAT-2 protein) converts the mono-unsaturated oleic acid to the di-unsaturated fatty acid linoleic acid. The delta-12 *fat2* RNAi prevents expression of the delta-12 fatty acid desaturase, which is predicted to cause a decrease in levels of linoleic acid in the nematode, leading to arrested development and death. Addition of 3 mM linoleic acid methyl ester to the NGM media used for the RNAi experiment brings about a partial rescue of the delta-12 *fat2* RNAi feeding phenotype. Addition of 3 mM oleic acid methyl ester does not rescue the delta-12 *fat2* RNAi feeding phenotype (see Table 1 below).

Table 1: *C. elegans* delta-12 *fat2* RNAi feeding phenotypes (starting with *C. elegans* L4 larvae as the P0 animal)

Fatty Acid Added	P0 phenotype	F1 phenotype	F2 phenotype
None	Reduced egg laying (partial sterility)	Developmentally arrested and sterile	NA
Oleic Acid Methyl Ester	Reduced egg laying (partial sterility)	Developmentally arrested and sterile	NA
Linoleic Acid Methyl Ester	Reduced egg laying	Moderately delayed development and moderately reduced egg laying	Slightly delayed development

Example 3

Preparation of *Caenorhabditis elegans* and Fatty Acids

Mixed stage *C. elegans* were washed off plates seeded with OP50 bacteria using M9 solution. 250 µl of the M9 solution, which contained about 50-100 worms, was pipetted into each well of a 24-well plate.

With the exceptions of the fatty acid salts and the free acid of ricinelaidic acid, all other fatty acid emulsions were prepared following the teachings of Kim *et al* (U.S. Patent Number 5,698,592). Briefly, 1 mL 1% stock solution emulsions were prepared by mixing 10 µl of fatty acid with 20 µl of the surfactant Igepal CO 630 in a 1.5 mL eppendorf tube. After careful mixing of fatty acid and Igepal CO 630, 850 µl of ddH_20 was added and mixed by gentle pipetting until a homogeneous solution was obtained. Finally, 120 µl of pure isopropanol was added and mixed by gentle pipetting. 1% stock emulsions were also prepared for the potassium salt of ricinoleic acid, the sodium salt of ricinelaidic acid, and ricinelaidic free acid. For the potassium salt of ricinoleic acid, 0.01 grams were dissolved in 100 µl of ddH_20, and combined with 20 µl of the surfactant Igepal CO 630 in a 1.5 mL eppendorf tube. After careful mixing of fatty acid and Igepal CO 630, 760 µl of ddH_20 was added and mixed by gentle pipetting until a homogeneous solution was obtained. Finally, 120 µl of pure isopropanol was added and mixed by gentle pipetting. For the sodium salt and free acid of ricinelaidic acid, 0.01 grams were dissolved in 100 µl of acetone, and combined with 20 µl of the surfactant Igepal CO 630 in a 1.5 mL eppendorf tube. After careful mixing

of fatty acid and Igepal CO 630, 760 µl of ddH_20 was added and mixed by gentle pipetting until a homogeneous solution was obtained. Finally, 120 µl of pure isopropanol was added and mixed by gentle pipetting. These stock solutions were then used to produce various fatty acid dilution emulsions in 24-well plate assays. An "acetone control" emulsion was prepared by combining 100 µl of acetone, 20 µl of the surfactant Igepal CO 630, 760 µl of ddH_20, and 120 µl of pure isopropanol in a 1.5 mL eppendorf tube and mixing to homogeneity.

Example 4

Nematicidal Activity of Single Fatty Acid Methyl Ester Emulsions Against *Caenorhabditis elegans*

To each well, fatty acid emulsions or control emulsions were added and rapidly mixed by swirling. Nematode viability was scored by visual observation and motility assays at various time points 24 hours following addition of emulsions or controls. The fatty acid emulsions tested were methyl esters of nonanoic (pelargonic) acid, ricinoleic acid, vernolic acid, linoleic acid, oleic acid, and control emulsions lacking fatty acids.

The structures of ricinoleic acid methyl ester, ricinelaidic acid methyl ester (not included in this table) and vernolic acid methyl ester are depicted in Fig. 1.

Table 2: Nematicidal activity of fatty acid methyl ester emulsions against *C. elegans*

Fatty Acid	Concentration	Percentage of Worm Death		
		1 hr	6 hr	24 hr
Nonanoic (C9-methyl ester)	0.1%	100%	100%	100%
	0.003%	50%	50%	50%
Ricinoleic Acid (C18-methyl ester)	0.1%	80%	80%	90%
	0.003%	40%	40%	40%
Vernolic Acid (C18-methyl ester)	0.1%	65%	65%	75%
	0.003%	20%	20%	20%
Linoleic Acid (C18-methyl ester)	0.1%	0-5%	0-5%	0-5%
	0.003%	0-5%	0-5%	0-5%
Oleic Acid (C18-methyl ester)	0.1%	0-5%	0-5%	0-5%
	0.003%	0-5%	0-5%	0-5%
Control (no methyl ester)	0.1%	0-5%	0-5%	0-5%
	0.003%	0-5%	0-5%	0-5%

Both nonanoic and ricinoleic acid methyl ester emulsions are strongly nematicidal at a concentration of 0.1%. Nonanoic methyl ester emulsions cause an almost immediate cessation of nematode movement and subsequent death whereas ricinoleic methyl ester emulsions require up to 30 minutes before strong killing effects are apparent. However, at 0.003%, nonanoic acid methyl ester emulsions temporarily "stunned" *C. elegans*, initially giving the appearance of a 100% death phenotype. Several hours post inoculation, many nematodes recover and start moving again. This "stun" effect was not observed with the other fatty acid emulsions.

Example 5

Nematicidal Activity of Single Fatty Acid Methyl Ester, Salt and Free Fatty Acid Emulsions Against *Caenorhabditis elegans* N2s and Dauers

L: linoleic acid, R: ricinoleic acid, Re: ricinelaidic; V-trans: (12,13)-epoxy-trans-9-octadecenoic acid; ME: methyl ester

Table 3: Results vs. *C. elegans* (worm death)

Fatty Acid	**0.1%**	**0.01%**	**0.001%**
Castor Oil	10%	<5%	NA
Pelargonic ME	100%	100%	30%
L ME	<5%	<5%	<5%
L free acid	10%	<5%	<5%
R ME	90%	40%	20%
R free acid	95%	50%	<5%
Re ME	100%	100%	80%
Re free acid*	100%	98%	40%
Potassium R	90%	15%	5%
Sodium Re*	100%	100%	NA
Acetone control	10%	5%	5%

Table 4: Results vs. *C. elegans* dauers (worm death)

Fatty Acid	**0.1%**	**0.01%**	**0.001%**
Castor Oil	NA	NA	NA
Pelargonic ME	NA	NA	NA
L ME	40%	20%	NA
L free acid	50%	40%	NA
R ME	70%	30%	NA
R free acid	90%	75%	NA
Re ME	100%	100%	NA
Re free acid*	75%	75%	NA
Potassium R	75%	20%	NA
Sodium Re*	NA	NA	NA
Acetone control	35%	20%	NA
V-trans ME	90%	50%	NA

Example 6

Preparation of Root Knot Nematode J2 Larvae (*Meloidogyne* spp.)

M. incognita and *M. javanica* were prepared from tomato roots. The roots were bleached and the debris was separated from the J2 larvae and eggs by filtration followed by sucrose density gradient centrifugation. Eggs were hatched over 4 days at 15 °C and the J2 larvae were collected by passage though a filter, followed by centrifugation.

Example 7

Nematicidal Activity of Fatty Acid Methyl Ester Emulsions Against Root Knot Nematodes (*Meloidogyne* spp.)

Nematodes and emulsions were incubated with shaking at room temperature for 48 hours. The contents of each well were transferred to a small spot on individual NGM plates lacking bacteria. About 24 hours after the transfer to plates, worms on and off the inoculation spot were counted as not viable or viable, respectively. Worms were considered viable if they had crawled away from the inoculation spot, or if they were moving. Worms were considered non-viable if they remained at the inoculation spot.

Table 5: Nematicidal activity of fatty acid methyl ester emulsions against *M. javanica* and *M. incognita*

Fatty acid (0.1%)	*M. javanica* (% not viable)	*M. incognita* (% not viable)
Vernolic Acid (C18-methyl ester)	90%	100%
Nonanoic (C9-methyl ester)	100%	100%
Ricinoleic Acid (C18-methyl ester)	60%	95%
Oleic Acid (C18-methyl ester)	20%	25%

Nonanoic, vernolic and ricinoleic acid methyl ester emulsions have significant nematicidal activity against root knot nematodes (*Meloidogyne* spp.) at a concentration of 0.1%.

Example 8

Phytotoxicity Evaluations of Fatty Acid Methyl Esters

Sterilized tomato seeds were germinated in magenta jars containing Gamborg's agar media. After two weeks of growth, seedlings were treated with 250 μl of 1% fatty acid methyl ester emulsion (nonanoic acid, ricinoleic acid, ricinelaidic acid, oleic acid, or a control emulsion lacking any fatty acid), applied directly to the stem-media interface. Tomato seedlings were scored at various times after application of emulsions. Of the fatty acids tested, only 1% nonanoic acid methyl ester emulsion showed obvious phytotoxic effects on the tomatoes. Within 18 hours of nonanoic acid emulsion application, those tomatoes showed a distinct loss of turgor pressure (wilting phenotype) and had become noticeably less green in appearance. Within 24 hours, nonanoic acid treated tomatoes were almost entirely bleached to a pale white color and had nearly totally collapsed with most leaves lying directly on the agar media surface. Importantly, none of the tomatoes treated with the other fatty acid methyl ester emulsions showed visible effects. Therefore, ricinoleic and ricinelaidic acid methyl esters show excellent potential as anthelmintic chemicals based on their combination of high nematicidal properties and with favorable low phytotoxicity.

Example 9

Nematicidal Activity of Single Fatty Acid Methyl Ester Emulsions Against a Spectrum of Free-Living, Animal Parasitic, and Plant Parasitic Nematodes

Briefly, the indicated fatty acid emulsions were added to nematodes in wells of a 24-well plate and rapidly mixed by swirling. Nematode viability was scored by visual observation and motility assays 24 hours following addition of emulsions (48 hours for plant parasitic nematodes *Meloidogyne* and *Heterodera* species). The fatty acid emulsions tested were methyl esters of nonanoic (pelargonic) acid, ricinelaidic acid, ricinoleic acid, vernolic acid, linoleic acid, and oleic acid. Results for fatty acid emulsions against free-living, animal parasitic, and plant parasitic nematodes are combined in one table to facilitate comparison of different emulsion activities against nematodes exhibiting diverse lifestyles. Results shown are mean % values obtained from multiple independent experiments

Table 6: Nematicidal activity of various fatty acid methyl esters against various free-living, animal parasitic, and plant parasitic nematodes

	% Worm Death (24 hr)					
	- control		Inhibitors			+ control
Worm (% solution)	Oleic	Linoleic	Vernolic	Ricinoleic	Ricinelaidic	Nonanoic
C. elegans (0.1%)	<10	<10	80	90	100	100
C. elegans (0.01%)	<10	<10	50	50	100	100
C. elegans (0.001%)		<10	30	30	75	30
P. trichosuri (0.1%)	~10	~25	~95	~50	100	
P. trichosuri (0.01%)	~10	~25	~90	~60	100	
P. trichosuri (0.001%)						
M. incognita (0.1%)		20	98	95	~99	100
M. incognita (0.01%)		20	73	83	~99	
M. incognita (0.001%)					97	
M. javanica (0.1%)		20	90	60	100	100
M. javanica (0.01%)		0-5	60	5	100	
M. javanica (0.001%)					~60	
H. glycines (0.1%)	<10	<20	30	~60	100	100
H. glycines (0.01%)	<10	<20	20	~60	100	>95
H. glycines (0.001%)	<10	<20	18	~40	100	
P. scribneri (0.1%)	<20	<20	<20	<20	~70	<20
P. scribneri (0.01%)	<20	<20	<20	<20	~40	<20
P. scribneri (0.001%)						

The *Caenorhabditis elegans* were mixed stage populations. Similar effects were seen on several other free-living nematode species. The *Parastrongyloides trichosuri* (parasite of Australian bushtail possum) were dauer-like infective 3rd stage larva. Similar effects are also seen against free-living stages. *The Meloidogyne incognita* and *Meloidogyne javanica* (root knot nematode) were 2nd stage juveniles (dauer-like infective stage). The *Heterodera glycines* (soybean cyst nematode) were 2nd stage juveniles (dauer-like infective stage). Finally, the *Pratylenchus scribneri* (corn lesion nematode) were mixed stage populations.

As the data in the table above demonstrate, both ricinelaidic and ricinoleic acid methyl ester emulsions are strongly nematicidal at concentrations of 0.1% and 0.01%.

Ricinelaidic acid methyl ester in particular showed favorable nematicidal activity against a wide spectrum of divergent nematode genera.

Example 10

The following table lists primers used in the cloning and preparation of various nucleic acids constructs including hydroxylases, epoxygenases, 5'-UTRs and 3'-UTRs.

Table 7: Sequence primers used in cloning

Name	Sequence	SEQ ID NO	Homology to
Hyd1	atgggaggtggtggtcgcatg	46	first 7 codons of *R. communis*
Hyd2	ttaatacttgttccggtacca	47	last 7 codons of *R. communis*
Les1	atgggtgctggtggaagaataatg	48	first 8 codons of *L. fendleri*
Les10	tcataacttattgaagtaatagtagacaccttt	49	last 11 codons of *L. fendleri*
les6	tcataacttattgttgtaata	50	last 7 codons of *L. fendleri*
Ecrep2	gcaatccctccccattg	51	codons 33-38 of *C. biennis*
Ecrep8	tcacaatttatcataccaataaacacc	52	last 9 codons of *C. biennis*
5'UTR-HIIIF	atacaaaagcttagagagagagattctgcgga	53	first 20 nt of *A. thaliana Fad2* 5' UTR
3'UTR-SphIR	attcaatgcatgcaacataatgagcagccaaaa	54	last 20nt of *A. thaliana Fad2* 3 UTR
Fad-HIIIF	attcaataagcttatgggtgcaggtggaagaat	55	first 7codons of *A. thaliana Fad2*
Fad-SphIR	atacaagcatgctcataacttattgttgtacc	56	last 7 codons of *A. thaliana Fad2*
3'Fad/cas	aagcaatggggtgggatggctttcttcagatctcccaccg	57	codons 31-38 Fad2/codons 43-49 *R. communis*
5'Fad/cas	cggtgggagatctgaagaaagccatcccaccccattgctt	58	codons 31-47 Fad2/codons 43-49 *R. communis*
Cas-SalR	gtcgacatacttgttccggtaccaga	59	last 7 codons of *R. communis*
3'Fad/les	cgattgctttcttcagatctcccaccgagaaaggcggtt	60	codons 28-33 Fad2/codons 35-41 *L. fendleri*
5'Fad/les	aaccgcctttctcggtgggagatctgaagaaagcaatcc	61	codons 28-33 Fad2/codons 35-41 *L. fendleri*
Les-SalIR	gtcgactaacttattgttgtaatagt	62	last 7AA of *L. fendleri*
3'Fad/lind	gggattgctttccttagatctcccaccgagaaaggcggtt	63	codons 28-33 Fad2/ codons 35-41 *L. lindheimeri*
5'Fad/lind	aaccgcctttctcggtgggagatctaaggaaagcaatccc	64	codons 28-33 Fad2/ codons 35-41 *L. lindheimeri*
Lind-SalIR	gtcgactaacttattgttgtaatagt	65	last 7 codons of *L. lindheimeri*
3'Fad/grac	aaccgcctttctcggtgggagatctgaagaaagcaatccc	66	codons 28-33 Fad2/ codons 35-41 *L. gracilis*
5'Fad/grac	gggattgctttcttcagatctcccaccgagaaaggcggtt	67	codons 28-33 Fad2/ codons 35-41 *L. gracilis*
Grac-SalIR	gtcgactcataacttattgttgtaat	68	last 7 codons of *L. gracilis*
3'Fad/crep	cggtgggagatctgaagaaagcaatccctccccattgctt	69	codons 32-38 Fad2/first 7 codons of partial *C. biennis*
5'Fad/crep	aagcaatggggagggattgctttcttcagatctcccaccg	70	codons 32-38 Fad2/first 7 codons of partial *C. biennis* clone
Crep-SalIR	gtcgaccaatttatgataccaataaa	71	last 7 codons of partial *C. biennis*

			clone
5'Castorhindlll-k	atacaaaagcttataatgggaggtggtggtcgcat	72	first 7 codons of *R. communis*
3' CastorBamH1	atacaaggatccttaatacttgttccggtacc	73	last 7 codons of *R. communis*
Castor-HANOTI	atacaagcggccgcagcgtaatctggaacatcgt	74	last 7 codons of *R. communis*
5'fendhindIII-K	atacaaaagcttataatgggtgctggtggaagaat	75	first 7 codons of *L. fendleri*
3' fendBamHI	atacaaggatcctcataacttattgttgtaat	76	first 7 codons of *L. fendleri*
5'HindIIIK/HA/fend	atacaaaagcttataatgtacccatacgatgttcc	77	first 7 codons of *L. fendleri*
UT3	atgagagctcgtttaaacgattttaatgtttagc	78	first 24 nt of UBI3 term
UT4	atgagaattcggccggccaatagtctcgac	79	last 20 nt of UBI3 term
UP1	tcatgaggcgcgccaaagcacatacttatcg	80	first 17 nt of UBI3 promoter
UP2	atgagcatgcaagcttcttcgcctggaggagag	81	last 23 nt of UBI3 promoter
HA5	agctatgtacccatacgatgttccagattacgctg	82	HA tag
HA6	tcgacagcgtaatctggaacatcgtatgggtacat	83	HA tag
CHA1	gatccatgtacccaatacgatgttccagattacgctctcgaggagct	84	HA tag
CHA2	ctcgagagcgtaatctggaacatcgtatgggtacatg	85	HA tag
IRT1	atgaggcgcgccctttctctgacttttaacatcc	86	first 22 nt of IRT2 promoter
IRT2	actggcatgcgtattgagattgttttataatatatg	87	last 26 nt of IRT2 promoter
Castor 5'HindIII	atacaaaagcttatgggaggtggtggtcgcat	88	first 6 codons of *R. communis*
Casotr 3'BamHI	atacaaggatccatacttgttccggtaccaga	89	last 6 codons of *R. communis*
fend F SalI	atacaaaagcttatgggtgctggtggaagaat	90	first 6 codons of L. *fendleri*
Fend R B-stop	atacaaggatcctaacttattgttgtaatagt	91	last 6 codons of L. *fendleri*
Castor 5' SalI	atacaagtcgacatgggaggtggtggtcgcat	92	first 6 codons of R. *communis*
Castor 3' BamH1	atacaaggatccatacttgttccggtaccaga	93	last 6 codons of R. *communis*
5'ΔKKGG2	ataaccagcaacaacagtgagagcagccaccttaagcgagc	94	codons 11-17, codons 22-27 of *R. communis*
3'ΔKKGG2	gctcgcttaaggtggctgctctcactgttgttgctggttat	95	codons 11-17, codons 22-27 of *R. communis*
5'ΔT	ttcttcctcagcctctctcttacctagcttggcctctctat	96	codons 76-82, codons 84-90 of *L. gracilis*
3'ΔT	atagagaggccaagctaggtaagagagaggctgaggaagaa	97	codons 76-82, codons 84-90 of *L. gracilis*
castor XbaI MfeI R	caattgtctagattaatacttgttccggtaccag	98	last 22 nt of *R. communis*
HIII NcoI castor F	aagcttaccatgggaggtggtggtcg	99	first 17 nt of *R. communis*
M13 Reverse	gaaacagctatgaccatg	100	M13 bacteriophage (M13/pUC plasmids)
gracilis XbaI MfeI R	caattgtctagatcataacttattgttgtaatag	101	last 22 nt of *L. gracilis*
HIII NcoI gracilis F	aagcttaccatgggtgctggtggaagaat	102	first 20 nt of *L. gracilis*
Crepis XbaI MfeI R	caattgtctagatcacaatttatgataccaataaa	103	last 23 nt of *C. biennis*
BamHI castor F	atacaaggatccaaatgggaggtggtggtcgcat	104	first 20 nt of *R. communis*
BamHI gracilis F	atacaaggatccaaatgggtgctggtggaagaat	105	first 20 nt of *L. gracilis*
BamHI NcoI S. epoxygenase F	aggatccctaccatgggtgcaggtggtcggat	106	first 20 nt of *S. laevis*
S. epoxygenase XbaI R	tctagattacatttttatggtaccagtaaa	107	last 20 nt of *S. laevis*
BglII NcoI C. biennis F	agatctctaccatgggtgcccacggccatgg	108	first 20 nt of *C. biennis*
HA-tag-F	agcttctcgagaccatggcgtacccgtacgacgtgcccgactacgccag	109	HA tag
HA-tag-R	gatcctggcgtagtcgggcacgtcgtacgggtacgccatggtctcgaga	110	HA tag
Fad5'UTR-F	atcctcgagagagattctgcggaggagcttc	111	*Fad2* 5' UTR of *A. thaliana*
Fad5'UTR-R	atcggatccatggttctgcagaaaaccaaaagca	112	*Fad2* 5' UTR of *A. thaliana*
Fad3'UTR-F	atctctagatgaggatgatggtgaagaaattg	113	*Fad2* 3' UTR of *A. thaliana*
Fad3'UTR-R	atcaagcttactgtccgaaggtcacatttc	114	*Fad2* 3' UTR of *A. thaliana*
Crep12F	ggaatgcatgtacatcgagcc	115	codons 355-360 of *C. biennis*
Crep13R	ggaacttgtgttggcatggtg	116	codons 138-144 of *C. biennis*
Estok-14	tggccngtntaytggttytg	117	codons 81-87 of *S. laevis*

Estok-17	tcyttngcytcyctccacat	118	codons 350-356 of *S. laevis*
Sl-1	atgggtgctggtggtcggatg	119	codons 1-7 of *S. laevis*
Stok-1R	gaacacgcttacacctaggac	120	codons 254-260 of *S. laevis*
Stok12R	atcaatccactggtattcac	121	codons 109-114 of *S. laevis*
Stok14F	gtcctaggtgtaagcgtg	122	codons 254-259 of *S. laevis*
HIII NcoI *C. biennis* F	aagcttaccatgggtgcccacggccatgg	123	first 20 nt of *C. biennis*
AscI NcoI *C. biennis F*	ggcgcgccaccatgggtgcccacggccatgg	124	first 20 nt of *C. biennis*

Example 11

The table below lists promoters and UTRs that can be used to achieve expression of polypeptides in plant vegetative tissue.

Table 8: Promoter-UTR sequences for genes strongly expressed in plant roots

Element	Species – Gene	Accession	Nucleotides
TobRB7	*Nicotiana tabacum* (common tobacco) – aquaporin	S45406	1 to 1953
TUB-1	*Arabidopsis thaliana* (thale cress) - beta 1-tubulin	M20405	1 to 569
PsMTA	*Pisum sativum* (pea) - metallothionein-like protein	Z23097	1 to 804
RPL16A	*Arabidopsis thaliana* (thale cress) - ribosomal protein L16	X81799	1 to 1014
ARSK1	*Arabidopsis thaliana* (thale cress) - serine/threonine protein kinase	L22302	1 to 807
AKT1	*Arabidopsis thaliana* (thale cress) – potassium transporter	U06745	1 to 231
LJAS2	*Lotus japonicus* - asparagine synthetase	X89410	1 to 144
MsH3g1	*Medicago sativa* - cultivar chief histone H3.2	U09458	1 to 482

Example 12

This example describes the cloning of delta-12 desaturase-like hydroxylases and epoxygenases (SEQ ID NO: 1 to 6 and 27 in sequence listings).

Cloning of Castor Oleate Hydroxylase Gene

Genomic DNA was isolated from *Ricinus communis* leaf tissue. Sense primer Hyd1 (SEQ ID NO: 46) and antisense primer Hyd2 (SEQ ID NO: 47) were used to amplify a genomic

copy of the castor hydroxylase gene in a Gradient PCR reaction [30 thermal cycles (1 min 95 °C, 30 sec 48-63 °C, 2 min 68 °C)] with KTLA DNA polymerase under standard conditions. The PCR product was fractionated in a 1% agarose gel. Bands approximately 1100 bp long were excised and gel purified (QIAquick Gel Extraction). DNA was cloned using a TOPO TA kit (Invitrogen). Candidate clones were sequenced in their entirety with an automated DNA sequencer (such as model 373 from Applied Biosystems, Inc.)

Cloning of *Lesquerella lindheimeri* and *Lesquerella gracilis* bifunctional hydroxylase genes

Genomic DNA was isolated from *L. lindheimeri* and *L. gracilis* leaf tissue. Sense primer Les1 (SEQ ID NO: 48) and antisense primer Les10 (SEQ ID NO: 49) were used to amplify genomic copies of both *Lesquerella* bifunctional hydroxylase genes in a PCR reaction [30 thermal cycles (2 min 94 °C, 1 min 55 °C, 2 min 68 °C)] with KTLA DNA polymerase under standard conditions. The PCR product was fractionated in a 1% agarose gel. Bands approximately 1100 bp long were excised and gel purified (QIAquick Gel Extraction). DNA was cloned using a TOPO TA kit (Invitrogen). Candidate clones were sequenced in their entirety with an automated DNA sequencer (such as model 373 from Applied Biosystems, Inc.)

Cloning of *Lesquerella fendleri* bifunctional hydroxylase gene

Genomic DNA was isolated from *L. fendleri*. Sense primer Les1 (SEQ ID NO: 48) and antisense primer Les6 (SEQ ID NO: 50) were used to amplify a genomic copy of the *L. fendleri* bifunctional hydroxylase gene in a Gradient PCR reaction [30 thermal cycles (1 min 95 °C, 30 sec 45-63 °C, 2 min 68 °C)] with KTLA DNA polymerase under standard conditions. The PCR product was fractionated in a 1% agarose gel. Bands approximately 1100 bp long were excised and gel purified (QIAquick Gel Extraction). DNA was cloned using a TOPO TA kit (Invitrogen). Candidate clones were sequenced in their entirety with an automated DNA sequencer (such as model 373 from Applied Biosystems, Inc.)

Cloning of *Crepis biennis* epoxygenase gene

Genomic DNA was isolated from *C. biennis*. Sense primer Ecrep2 (SEQ ID NO: 51) and antisense primer Ecrep8 (SEQ ID NO: 52) were used to amplify a partial genomic clone of

the *C. biennis* epoxygenase gene in a Gradient PCR reaction [30 thermal cycles (1 min 95 °C, 30 sec 45-63 °C, 2 min 68 °C) with KTLA DNA polymerase under the standard conditions]. The PCR product was fractionated on a 1% agarose gel and a band approximately 1100 bp long was excised and gel purified (QIAquick Gel Extraction). The gene fragment was then cloned using a TOPO TA cloning kit (Invitrogen). Candidate clones were sequenced in their entirety with an automated DNA sequencer (such as model 373 from Applied Biosystems, Inc.) to yield plasmid clone, Div2966. Partial sequence data for the *C. biennis* epoxygenase was obtained from Div2966, including nucleotide sequence for codons 33-374 and the 3' stop codon. The clone lacked the first 32 codons of the *C. biennis* epoxygenase, as well as the 5' untranslated region. To obtain the missing 5' sequence of the *C. biennis* epoxygenase gene, the inverse PCR technique was applied. Inverse PCR permits the rapid amplification of unknown segments of DNA that immediately flank a target sequence. Briefly, *C. biennis* genomic DNA is digested with a selected restriction enzyme, then ligated to circularize smaller segments of genomic DNA. These circularized segments are then used as templates for PCR with primers directing DNA amplification outward away from the known region of the gene of interest to amplify the missing flanking sequences. Inverse PCR can be used to amplify missing 5' or 3' sequences. The digested, ligated, and circularized genomic DNA was directly PCR amplified using gene-specific primers (Crep12F; SEQ ID NO: 115 and Crep13R; SEQ ID NO: 116) designed from the known sequence that anneal within the gene of interest. This procedure was performed to generate clone Div4373, which contains codons 1-137 and 355-374. Taken together, clone Div2966 and Div4373 contain sequences comprising the complete open reading frame of the epoxygenase gene of *C. biennis*.

Cloning of *Stokesia leavis* epoxygenase gene

Genomic DNA was isolated from *S. laevis*. Degenerate primers were designed to anneal to regions within the *S. leavis* epoxygenase gene which were predicted to exhibit a high degree of sequence conservation across many plant epoxygenases. Sense primer Estok14 (SEQ ID NO: 117) and antisense primer Estok17 (SEQ ID NO: 118) were used to amplify a genomic fragment of the *S. laevis* epoxygenase gene. Amplified PCR products were then cloned into a suitable vector for DNA analysis. This procedure was performed to obtain clone Div4023.

This clone contained codons 88-356. To obtain the 5'end sequence of the gene, gene-specific primers designed from known sequence that anneal within the gene of interest, sense primer Sl-1 (SEQ ID NO: 119) and antisense primer Stok1R (SEQ ID NO: 120), were used to amplify the rest of the epoxygenase gene. This yielded plasmid clone Div4172. This clone contained codons 1-260. To obtain the 3' end of the *S. laevis* epoxygenase gene, the inverse PCR technique was applied. Inverse PCR permits the rapid amplification of unknown segments of DNA that immediately flank a target sequence. Briefly, *S. laevis* genomic DNA is digested with a selected restriction enzyme, then ligated to circularize smaller segments of genomic DNA. These circularized segments are then used as templates for PCR with primers directing DNA amplification outward away from the known region of the gene of interest to amplify the missing flanking sequences. Inverse PCR can be used to amplify missing 5' or 3' sequences. The digested, ligated, and circularized genomic DNA was directly PCR amplified using gene-specific primers, Stok12R (SEQ ID NO: 121) and Stok14F (SEQ ID NO: 122), designed from the known sequence that anneal within the gene of interest. This procedure was performed to generate clone Div4324, which contains codons 1-108 and 254-377. Taken together, clone Div4023, Div4172 and Div4324 contain sequences comprising the complete open reading frame of the epoxygenase gene of *S. laevis*.

Cloning of ΔT *L. gracilis* bifunctional hydroxylase construct: Specific primers were designed to remove nucleotides 245-247 (CTA) from the full length *R. communis* hydroxylase gene. A two-round PCR based subcloning strategy was used to generate the ΔT *L. gracilis* bifunctional hydroxylase. The first round of PCR primers were as follows; to amplify 5' end of the bifunctional hydroxylase excluding nucleotides 245-247, the sense primer M13 Reverse (SEQ ID NO: 100) and antisense primer 3'ΔT (SEQ ID NO: 97) were used in a PCR reaction using a copy of the *L. gracilis* bifunctional hydroxylase gene contained in the cloning vector pCR2.1 as a template. To amplify the 3' end of the bifunctional hydroxylase gene excluding nucleotides 245-247, the sense primer 5'ΔT (SEQ ID NO: 96) and antisense primer gracilis XbaI MfeI R (SEQ ID NO: 101) were used. For the second round of PCR, sense primer HIII NcoI gracilis F (SEQ ID NO: 102) and gracilis XbaI Mfe R (SEQ ID NO: 101) were used to generate the final PCR product ΔT *L. gracilis* hydroxylase. PCR products were amplified using 5 thermal cycles (1 min, 94 C, 30 sec 50 °C, 1.5 min 68 °C) and then 15 thermal cycles (1 min, 94 °C, 30 sec 57 °C, 1.5 min 68 °C)

with KTLA DNA polymerase under standard conditions. The construct was then subcloned into a plant expression vector using the *Nco*I and *Xba*I restriction enzymes sites.

Cloning of the ΔKKGG *Ricinus communis* hydroxylase construct: Specific primers were designed to remove nucleotides 53-64 (AGAAAGGAGGAA, SEQ ID NO: 130) from the full length *R. communis* hydroxylase gene. A two-round PCR based subcloning strategy was used to generate the ΔKKGG *Ricinus communis* hydroxylase gene. The first round of PCR primers were as follows; to amplify 5' end of the *Ricinus* hydroxylase gene excluding nucleotides 53-64, the sense primer M13 Reverse (SEQ ID NO: 100) and antisense primer 3'ΔKKGG2 (SEQ ID NO: 95) were used in a PCR reaction using a copy of the *R. communis* hydroxylase gene contained in the cloning vector pCR2.1 as a template. To amplify the 3' end of the *Ricinus* hydroxylase gene excluding nucleotides 53-64, the sense primer 5'ΔKKGG2 (SEQ ID NO: 94) and antisense primer castor XbaI MfeI R (SEQ ID NO: 98) were used. For the second round of PCR, sense primer HIII NcoI castor F (SEQ ID NO: 99) and castor XbaI Mfe R (SEQ ID NO: 98) were used to generate the final PCR product ΔKKGG *Ricinus communis* hydroxylase. PCR products were amplified using 5 thermal cycles (1 min, 94 °C, 30 sec 50 C, 1.5 min 68 C) and then 15 thermal cycles (1 min, 94 C, 30 sec 57 C, 1.5 min 68 C) with KTLA DNA polymerase under standard conditions. The construct was then subcloned into a plant expression vector using the *Nco*I and *Xba*I restriction enzymes sites.

Example 13

This example describes the isolation of the *Arabidopsis thaliana fad2* regulatory and coding sequences and the construction of *fad2*/hydroxylase and *fad2*/epoxygenase fusion polypeptides. See SEQ ID NO: 7 to 12 in the sequence listings.

Isolation of the *A. thaliana fad2* desaturase cDNA clone

Total RNA was isolated from *A. thaliana* leaf tissue (Qiagen RNeasy). RT-PCR was performed using the Roche Titan One Tube RT-PCR system with sense primer 5'UTR-HIIIF (SEQ ID NO: 53) and antisense primer 3'UTR-SphIR (SEQ ID NO: 54). RT-PCR was set up following the kit directions [1 cycle (30 minutes 50 °C), 1 cycle (2 minutes 94 °C), 10

cycles (10 seconds 94 °C, 30 seconds 60 °C, 1 minute 68 °C), 25 cycles (10 seconds 94 °C, 30 sec 60 °C, 1 min 68 °C + cycle elongation of 5 seconds for each cycle), 1 cycle (7 min 68 °C)]. Bands approximately 1100bp long were excised and gel purified (QIAquick Gel Extraction). DNA was cloned using a TOPO TA kit (Invitrogen). Candidate clones were sequenced in their entirety with an automated DNA sequencer (such as model 373 from Applied Biosystems, Inc.)

Isolation of the *A. thaliana* fad2 desaturase genomic DNA clone

Genomic DNA was isolated from *A. thaliana* leaf tissue. Sense primer 5'UTR-HIIIF (SEQ ID NO: 53) and antisense primer 3'UTR-SphIR (SEQ ID NO: 54) were used to amplify genomic *fad2* DNA in a PCR reaction [5 thermal cycles (1 min 95 °C, 30 sec 54 °C, 2 min 68 °C), 25 thermal cycles (1 min 95 °C, 30 sec 62 °C, 2 min 68 °C)] with KTLA DNA polymerase under the standard conditions. The PCR product was fractionated in a 1% agarose gel. Bands approximately 2400bp long were excised and gel purified (QIAquick Gel Extraction). DNA was cloned using a TOPO TA kit (Invitrogen). Candidate clones were sequenced in their entirety with an automated DNA sequencer (such as model 373 from Applied Biosystems, Inc.)

Generation of *fad2/Ricinus communis* hydroxylase chimeric cDNA

A two-round PCR based subcloning strategy was used to generate all of the chimeric cDNAs. In the first round of PCR, sense primer Fad-HIIIF (SEQ ID NO: 55) and antisense primer 3'Fad/cas (SEQ ID NO: 57) were used to amplify the first 114 bases from the *fad2* cDNA clone. Sense primer 5'-Fad/cas (SEQ ID NO: 58) and antisense primer Cas-SalR (SEQ ID NO: 59) were used to amplify the last 1034 bases (excluding TAA) of the *Ricinus communis* hydroxylase cDNA clone by PCR [1 thermal cycle (4 min 94 °C), 5 thermal cycles (45 sec 94 °C, 45 sec 50 °C, 60 sec 68 °C), 25 thermal cycles (45 sec 94 °C, 45 sec 57 °C, 60 sec 68 °C) with KTLA DNA polymerase under the standard conditions. The PCR products were fractionated on a 1% agarose gels. The bands were excised and cleaned (QIAquick Gel Extraction – final volume 50 uL). The clean product was diluted 1:100 (TE) and both DNAs were used as the template (1 μL each) in the second round of PCR. In the second round of PCR sense primer Fad-HIIIF (SEQ ID NO: 55) and antisense primer Cas-SalR (SEQ ID NO:

59) were used to generate the final PCR product *fad2/Ricinus communis* chimeric cDNA [1 thermal cycle (4 min 94 °C), 5 thermal cycles (45 sec 94 °C, 45 sec 50 °C, 60 sec 68 °C), 25 thermal cycles (45 sec 94 °C, 45 sec 57 °C, 60 sec 68 °C) with KTLA DNA polymerase under standard conditions]. A band approximately 1300 bp long was excised and gel purified (QIAquick Gel Extraction). DNA was cloned using a TOPO TA kit (Invitrogen). Candidate clones were sequenced in their entirety with an automated DNA sequencer (such as model 373 from Applied Biosystems, Inc.)

Generation of *fad2/Lesquerella fendleri* hydroxylase chimeric cDNA

The same two-round PCR based subcloning strategy was used to generate the *fad2/Lesquerella fendleri* chimeric cDNA. The first round PCR primers were as follows; to amplify the 5' end of the *A. thaliana fad2*, the sense primer Fad-HIIIF (SEQ ID NO: 55) and antisense primer 3'-Fad/les (SEQ ID NO: 60) were used. To amplify the 3' end of the *L. fendleri* bifunctional hydroxylase gene, sense primer 5'Fad/les primer (SEQ ID NO: 61) and antisense primer Les-SalIR (SEQ ID NO: 62) were used. In the second round of PCR, sense primer Fad-HIIIF (SEQ ID NO: 55) and antisense primer Les-SalIR (SEQ ID NO: 62) were used to generate the final PCR product *fad2/Lesquerella fendleri* chimeric cDNA.

Generation of *fad22/Lesquerella lindheimeri* hydroxylase chimeric cDNA

The same two-round PCR based subcloning strategy was used to generate the *fad2/Lesquerella lindheimeri* chimeric cDNA. The first round of PCR primers were as follows; to amplify the 5' end of the *A. thaliana fad2*, the sense primer Fad-HIIIF (SEQ ID NO: 55) and antisense primer 3'-Fad/lind (SEQ ID NO: 63) were used. To amplify the 3' end of the *L. lindheimeri* bifunctional hydroxylase gene, sense primer 5'Fad/lind primer (SEQ ID NO: 64) and antisense primer Lind-SalIR (SEQ ID NO: 65) were used. In the second round of PCR, sense primer Fad-HIIIF (SEQ ID NO: 55) and antisense primer Lind-SalIR (SEQ ID NO: 65) were used to generate the final PCR product *fad2/Lesquerella lindheimeri* chimeric cDNA.

Generation of *fad2/Lesquerella gracilis A* hydroxylase chimeric cDNA

The same two-round PCR based subcloning strategy was used to generate the *fad2/Lesquerella gracilis A* chimeric cDNA. The first round of PCR primers were as follows; to amplify the 5' end of the *A. thaliana fad2*, the sense primer Fad-HIIIF (SEQ ID NO: 55) and antisense primer 3'-Fad/grac (SEQ ID NO: 66) were used. To amplify the 3' end of the *L. gracilis* bifunctional hydroxylase gene, sense primer 5'-Fad/grac primer (SEQ ID NO: 67) and antisense primer Grac-SalIR (SEQ ID NO: 68) were used. In the second round of PCR, sense primer Fad-HIIIF (SEQ ID NO: 55) and antisense primer Grac-SalIR (SEQ ID NO: 68) were used to generate the final PCR product *fad2/Lesquerella gracilis* A chimeric cDNA.

Generation of *fad2/Crepis biennis* epoxygenase chimeric cDNA

The same two-round PCR based subcloning strategy was used to generate the *fad2/Crepis biennis* chimeric cDNA. The first round of PCR primers were as follows; to amplify the 5' end of the *A. thaliana fad2*, the sense primer Fad-HIIIF (SEQ ID NO: 55) and antisense primer 3'-Fad/crep (SEQ ID NO: 69) were used. To amplify the 3' end of the *C. biennis* epoxygenase, sense primer 5'Fad/crep primer (SEQ ID NO: 70) and antisense primer Crep-SalIR (SEQ ID NO: 71) were used. The second round of PCR sense primer Fad-HIIIF (SEQ ID NO: 55) and antisense primer Crep-SalIR (SEQ ID NO: 71) were used to generate the final PCR product *fad2/Crepis biennis* chimeric cDNA.

Example 14

This example describes the construction of six synthetic optimized hydroxylase and epoxygenase sequences.

Four codon optimized hydroxylase (*Ricinus communis* and *Lesquerella gracilis*) and epoxygenase (*Stokesia laevis* and *Crepis bienis*) sequences were constructed as follows. First the 2nd, 3rd, and 4th codons downstream of the initiation methionine codon were changed to GCT, TCC, and TCC (encoding alanine, serine and serine). Secondly, codons with 8% or lower percentage occurrence in either the *Arabidopsis thaliana*, *Glycine max*, *Lycopersicon esculentum* or *Nicotiana tabacum* genomes (e.g., CGG for arginine) were replaced with the most frequent or second most frequent codon for that particular amino acid

(e.g., AGA or AGG for arginine). Finally, one member of a contiguous pair of codons was optimized if both codons had an occurrence of 12% or lower in either the *Arabidopsis thaliana*, *Glycine max*, *Lycopersicon esculentum* or *Nicotiana tabacum* genomes. Data for the codon optimization process were taken from the codon usage database (http://www.kazusa.or.jp/codon/).

Codons were also changed to remove ATTTA (i.e., AUUUA) elements which may destabilize mRNAs, to ablate potential polyadenylation sites, and to break up runs of A, G, C or T of five or greater nucleotides (e.g., TTTTT). Codons were also modified to reduce the likelihood of aberrant splicing. Splicing potential was assessed with the NetPlantGene prediction server (http://www.cbs.dtu.dk/services/NetPGene/). Whenever a donor and acceptor existed where both were predicted with greater than 0.9 confidence a codon was mutated to ablate either the donor (GT) or acceptor (AG) sites and thus diminish splicing potential. SEQ ID NOS: 30, 31, 32 and 33 are examples of these optimized sequences.

Additional codon optimized variants of the *Ricinus communis* hydroxylase and *Stokesia laevis* epoxgenase genes were made. These additional sequences contained modifications to more closely mimic the most common soybean (*Glycine max*) codons. The 2nd, 3rd, and 4th codons downstream of the initiation methionine codon were changed to GCT, TCC, and TCC (encoding alanine, serine and serine). Codons were also changed to remove ATTTA (i.e., AUUUA) elements which may destabilize mRNAs, to ablate potential polyadenylation sites, and to break up runs of A, G, C or T of five or greater nucleotides (e.g., TTTTT). Codons were also modified to reduce the likelihood of aberrant splicing. Splicing potential was assessed with the NetPlantGene prediction server (http://www.cbs.dtu.dk/services/NetPGene/). Whenever a donor and acceptor existed where both were predicted with greater than 0.9 confidence a codon was mutated to ablate either the donor (GT) or acceptor (AG) sites and thus diminish splicing potential. Data for codon optimization procedures were taken from the codon usage database (http://www.kazusa.or.jp/codon/). SEQ ID NOS: 28 and 29 are examples of such optimized *R. communis* and *S. laevis* hydroxylase and epoxygenase genes, respectively.

Example 15

This example describes the expression of hydroxylase, bifunctional hydroxylase and epoxygenase polypeptides in *Saccharomyces cerevisiae* and analysis of the fatty acid profiles in yeast by GC-MS.

Yeast Stains, Media, and Culture Conditions

Saccharomyces cerevisiae strains YPH499 (MATa ura3-52 lys2-801 ase2-101 trp1-Δ63 his3-Δ2000 leu2-Δ1) and INVsc1 (MATa his3-Δ1 leu2 trp1-289 ura3-52/MATα his3Δ1 leu2 trp1-289 ura3-52) were used throughout these studies.

Plasmid for Yeast Transformation

The plasmid pYES2 (Invitrogen) was used to transform yeast strains. The plasmid contains an *E. coli* replication origin, a yeast plasmid replication origin, an *E. coli* ampicillin resistance gene and the yeast gene URA3. It utilizes an expression cassette including a galactose-inducible promoter (GAL-1).

Cloning Genes of Interest into Yeast Expression Vector pYES2

Modification of the *R. communis* hydroxylase and *L .gracilis* bifunctional genomic clones were performed by PCR amplification using specific primers.

Ricinus communis hydroxylase: Specific primers were designed to introduce a Kozak consensus sequence and a *Hind*lll restriction site immediately upstream of the initiation codon and a *Bam*H1 site immediately downstream of the stop codon. Direct primer: 5'-CastorhindIII-k (SEQ ID NO: 72). Reverse primer: 3'CastorBamHI. (SEQ ID NO: 73). The hydroxylase was amplified by PCR [5 thermal cycles (1 min, 92 C, 30 sec 50 C, 1.5 min 68 C) and then 25 thermal cycles (1 min, 92 C, 30 sec 57 C, 1.5 min 68 C) with KTLA DNA polymerase under standard conditions]. The PCR product was digested with *Hind*lll and *Bam*H1 and subsequently cloned into *Hind*lll, *Bam*H1 of pYES2 yeast expression vector.

Ricinus communis hydroxylase with a C-terminal HA tag: Specific primers were designed to introduce a Kozak consensus sequence and a *Hind*lll site immediately upstream of the start codon and a *Not*I site and HA tag immediately before the stop codon. Direct

primer: 5'-castorhindIII-k (SEQ ID NO: 72). The Reverse primer: 5'-castor-HANOTI (SEQ ID NO: 74). The hydroxylase with a C-terminal HA tag was amplified by PCR [5 thermal cycles (1 min, 92 C, 30 sec 50 C, 1.5 min 68 C) and then 25 thermal cycles (1 min, 92 C, 30 sec 57 °C, 1.5 min 68 C) with KTLA DNA polymerase under standard conditions]. The PCR product was digested with *Hind*III and *Not*I and subsequently cloned into the *Hind*III, *Not*I sites of the pYES2 expression vector.

Ricinus communis hydroxylase with a N-terminal HA tag: Direct primer: BamHI castor F (SEQ ID NO: 104). Reverse primer: castor XbaI MfeI R(SEQ ID NO: 98). The hydroxylase was amplified by PCR [5 thermal cycles (1 min, 92 C, 30 sec 50 C, 1.5 min 68 C) and then 25 thermal cycles (1 min, 92 C, 30 sec 57 C, 1.5 min 68 C) with KTLA DNA polymerase under standard conditions]. The PCR product was digested with *BamHI /MfeI* and subcloned into the *BamHI / EcorI* sites of the pUC-HA vector. The hydroxylase plus the n-terminal HA tag was then subcloned (HindIII/XbaI) into the yeast expression vector pYES2.

Lesquerella lindheimeri bifunctional enzyme: Specific primers were designed to introduce a Kozak consensus sequence and a *Hind*III restriction site immediately upstream of the initiation codon and a *Bam*H1 site immediately downstream of the stop codon. Direct primer: 5'-fendhindIII-K (SEQ ID NO: 75). Reverse primer: 3'-fendBamHI (SEQ ID NO: 76). The hydroxylase was amplified by PCR [5 thermal cycles (1 min, 92 C, 30 sec 50 C, 1.5 min 68 C) and then 25 thermal cycles (1 min, 92 C, 30 sec 57 C, 1.5 min 68 C) with KTLA DNA polymerase under standard conditions]. The PCR product was digested with *Hind*III, *Bam*H1 and cloned into the *Hind*III, *Bam*H1 of pYES2 yeast expression vector.

Lesquerella lindheimeri bifunctional enzyme with a N-terminal HA tag: Specific primers were designed to introduce a Kozak consensus sequence and a *Hind*III site immediately upstream of the HA tag and a *Bam*H1 site immediately before the stop codon. Direct primer 5'-HindIIIK/HA/fend (SEQ ID NO: 77). Reverse primer: 3'-fendBamHI (SEQ ID NO: 76) The hydroxylase with a N-terminal HA tag was amplified by PCR [5 thermal cycles (1 min, 92 C, 30 sec 50 C, 1.5 min 68 C) and then 25 thermal cycles (1 min, 92 C, 30 sec 57 C, 1.5 min 68 C) with KTLA DNA polymerase under standard conditions]. The PCR product

was digested with *Hind*III and *Bam*H1 and subsequently cloned into *Hind*III, *Bam*H1 of pYES2 expression vector.

Lesquerella gracilis bifunctional enzyme: Specific primers were designed to introduce a Kozak consensus sequence. Direct primer: HIII NcoI gracilis F (SEQ ID NO: 102). Reverse primer: gracilis XbaI MfeI R (SEQ ID NO: 101). The hydroxylase was amplified by PCR [5 thermal cycles (1 min, 92 C, 30 sec 50 C, 1.5 min 68 C) and then 25 thermal cycles (1 min, 92 C, 30 sec 57 C, 1.5 min 68 C) with KTLA DNA polymerase under standard conditions]. The PCR product was digested with *Hind*III, *XbaI* and cloned into the *Hind*III, *XbaI* of pYES2 yeast expression vector.

Δ*T Lesquerella gracilis* bifunctional enzyme: Specific primers were designed to introduce a Kozak consensus sequence. Direct primer: HIII NcoI gracilis F (SEQ ID NO: 102). Reverse primer: gracilis XbaI MfeI R (SEQ ID NO: 101). The Δ*T L. gracilis* hydroxylase was amplified by PCR [5 thermal cycles (1 min, 92 C, 30 sec50 C, 1.5 min 68 C) and then 25 thermal cycles (1 min, 92 C, 30 sec 57 C, 1.5 min 68 C) with KTLA DNA polymerase under standard conditions]. The PCR product was digested with *Hind*III, *XbaI* and cloned into the *Hind*III, *XbaI* of pYES2 yeast expression vector.

Δ*KKGG Ricinus communis* hydroxylase: Specific primers were designed to introduce a Kozak consensus sequence. Direct primer: HIII NcoI castor F (SEQ ID NO: 99). Reverse primer: castor XbaI MfeI R (SEQ ID NO: 98). The hydroxylase was amplified by PCR [5 thermal cycles (1 min, 92 C, 30 sec 50 C, 1.5 min 68 C) and then 25 thermal cycles (1 min, 92 C, 30 sec 57 C, 1.5 min 68 C) with KTLA DNA polymerase under standard conditions]. The PCR product was digested with *Hind*III, *XbaI* and cloned into the *Hind*III, *XbaI* of pYES2 yeast expression vector.

Crepis biennis epoxygenase enzyme: Specific primers were designed to introduce a Kozak consensus sequence. Direct primer: HIII NcoI *C. biennis* F (SEQ ID NO: 123). Reverse primer: *Crepis* XbaI MfeI R (SEQ ID NO: 103). The hydroxylase was amplified by PCR [5 thermal cycles (1 min, 92 C, 30 sec 50 C, 1.5 min 68 C) and then 25 thermal cycles (1 min,

92 C, 30 sec 57 C, 1.5 min 68 °C) with KTLA DNA polymerase under standard conditions]. The PCR product was digested with *Hind*lll, *XbaI* and cloned into the *Hind*lll, *XbaI* of pYES2 yeast expression vector.

Stokesia laevis epoxygenase enzyme: Specific primers were designed to introduce a Kozak consensus sequence. Direct primer: BamHI NcoI S. epoxygenase F (SEQ ID NO: 106). Reverse primer: S. epoxygenase XbaI R (SEQ ID NO: 107). The hydroxylase was amplified by PCR [5 thermal cycles (1 min, 92 C, 30 sec 50 C, 1.5 min 68 C) and then 25 thermal cycles (1 min, 92 °C, 30 sec 57 C, 1.5 min 68 C) with KTLA DNA polymerase under standard conditions]. The PCR product was digested with *BamHI, XbaI* and cloned into the *BamHI, XbaI* of pYES2 yeast expression vector.

Nucleotide Sequence Determination

Sequencing of the *R. communis* hydroxylase, *R. communis* hydroxylase with N-terminal HA tag, *R. communis* hydroxylase with C-terminal HA tag, *L. lindheimeri* bifunctional enzyme, *L. lindheimeri* bifunctional enzyme with N-terminal HA tag, ΔT *L. gracilis*, and *ΔKKGG R. communis* hydroxylase were performed using an automated sequencer (such as model 373 from Applied Biosystems, Inc.) using processes well known to those skilled in the art.

Transformation of Yeast

Transformation was preformed according to Invitrogen pYES2 kit (V825-20). A fresh yeast culture (initial absorbance = 0.4) was grown in YPD medium for 4 hours. The cells were collected and washed once in 1X TE and resuspended in 2 mL of 1X LiAc/0.5X TE (100mm lithium acetate pH 7.5, 5mm tris-HCL pH 7.5, 0.5mm EDTA). 100 μg of denatured herring sperm DNA was added as a DNA carrier to 1 μg of the plasmid DNA. 100 μL of competent yeast and 700 μL of 1XliAc/40%PEG-3350/1XTE (100mM lithium acetate pH 7.5, 40% PEG-3350, 10 mM tris-HCL pH 7.5, 1 mM EDTA) were added. The mixture was incubated at 30 °C for 30 min. 88 μL of DMSO was added and the mixture was incubated at 42 °C for 7 min. After centrifugation, the cells were resuspended in 1X TE (100 uL) and plated on minimum medium containing suitable supplements.

Over Expression of Genes of Interest in Yeast

Yeast strains transformed with pYES2 plasmid, harboring either no insert or the genes for hydroxylase or bifunctional enzymes were always grown at the same time. For ricinoleic acid analysis, transformed cells were grown in SC-URA (yeast synthetic complete media devoid uracil, Sigma) supplemented with 2% glucose and 1% casamino acids at 30 °C to an optical density (600nm) of 2.5. Cells were then centrifuged, washed 3 times in SC-URA media containing no glucose and cultured for 48 hours at 30 °C on SC-URA media (yeast synthetic complete media devoid of uracil, Sigma) supplemented with 2% galactose and 1% casamino acids. Cultures were centrifuged and dried.

Fatty Acid Analysis of yeast extracts

Dried yeast pellets were methylated with (400 µL 1% sodium methoxide in methanol), extracted with hexane, and trimethylsilylated (100 µL BSTAFA-TMCS, Supelco, 90 °C for 45 minutes). Samples were analyzed on an Agilent 6890 GC-5973 Mass Selective Detector (GC/MS) and an Agilent DB-23 capillary column (0.25 mm x 30 m x 0.25 um). The injector was held at 250 °C, the oven temperature was 235 °C, and a helium flow of 1.0 mL/min was maintained.

Table 9 shows examples of MS data from yeast expressing some of the enzymes described in Example 13.

Table 9: *Ricinus communis* hydroxylase with or without an N-terminal HA tag:

Construct	%R
3522	6.7
3522	4.1
3522	4.8
3522	9.5
3522	4.6
4074*	2.1
4074*	3.0
4074*	5.3
4074*	3.2

*Designates a construct carrying an N-terminal HA tag.

These GC/MS data indicate that the hydroxylase from *R. communis* (3522 or 4074*) was functional when expressed in yeast. The percentages of ricinoleic acid (%R) listed in the table are percentages of the total fatty acid.

Table 10: *L. gracilis* bifunctional hydroxylase expressed in yeast

Construct	%R
3958	8.0
3958	8.2
3958	13.1
3958	12.2
3958	10.7
3958	9.2
3958	6.3

These GC/MS data indicate that the hydroxylase from *L. gracilus* (3958) was functional when expressed in yeast. The percentages of ricinoleic acid (%R) listed in the table are percentages of the total fatty acid.

Table 11: ΔT *Lesquerella gracilis* bifunctional hydroxylase expressed in yeast

Construct	%R
4323	5.9
4323	5.9
4323	8.2
4323	7.2
4323	7.4

These GC/MS data indicate that the hydroxylase from *L. gracilis* was functional when expressed in yeast despite the deletion of amino acid 83. The percentages of ricinoleic acid (%R) listed in the table are percentages of the total fatty acid.

Table 12: ΔKKGG castor hydroxylase expressed in yeast

Construct	%R
4303	0.7
4303	1.4
4303	1.5
4303	1.3
4303	1.5

These GC/MS data indicate that the deletion mutant hydroxylase (ΔKKGG) from *R. communis* was functional when expressed in yeast despite the amino acid deletions at positions 18-21. The percentages of ricinoleic acid (%R) listed in the table are a percentage of the total fatty acid.

Table 13: Negative Control

Construct	%R	%O
3677	0	35.8
3677	0	34.71
3677	0	36.34
3677	0	30.87
3677	0	30.16

These GC/MS data indicate that no detectable amounts of ricinoleic acid were produced when the vector with no insert was expressed in yeast. The percentages of ricinoleic (%R) and oleic acid (%O) listed in the table are percentage of the total fatty acid.

Example 16

This example describes the construction of vectors suitable for expression in plants. Schematic diagrams of the vectors are shown in Figures 4-6.

Generation of Transgenic Vectors: Building Modified pUCAP Vectors

The pUCAP vector [Engelen et al. (1995) *Transgenic Res.* 4(4):288-290] was modified to create pUCAP2, pUCAP3, pUCAP4, pUCAP5, and pUCAP6.

Specific primers were designed to introduce a 5'-*Sac*I and a 3'-*Eco*RI site flanking the Ubi3 terminator. Direct primer: UT3 (SEQ ID NO: 78). Reverse primer: UT4 (SEQ ID NO: 79). The Ubi3 terminator was amplified from pBinplus [Engelen et al. (1995) *Transgenic Res.* 4(4):288-290] by PCR [25 cycles (4 min 94 °C, 30 sec 60 °C, 1 min 68 °C) with KTLA DNA polymerase under standard conditions]. The PCR product was digested with *Sac*I and *Eco*RI and subsequently cloned into pUCAP to give pUCAP1.

Specific primers were designed to introduce a 5'-AscI and a 3'-SphI site flanking the Ubi 3 promoter. Direct primer: UP1 (SEQ ID NO: 80). Reverse primer: UP2 (SEQ ID NO: 81). The Ubi3 promoter was amplified from pBinplus [Engelen et al. (1995) *Transgenic Res.* 4(4):288-290] by PCR [25 cycles (4min 94 °C, 30 sec 60 °C, 1 min 68 °C) with KTLA DNA polymerase under standard conditions]. The PCR product was digested with AscI and SphI and subsequently cloned into the AscI/SphI sites of pUCAP1 giving pUCAP2.

Specific oligos were designed to create an HA tag with a *Bam*H1 overhang immediately before the initiation codon and a *Sac*I overhang immediately after the last codon of the tag. Direct oligo: CHA1 (SEQ ID NO: 84). Reverse oligo: CHA2 (SEQ ID NO: 85). The HA tag was created by annealing oligos (0.1 pg/uL) at 92 °C for 3 minutes and slowly bringing to room temperature. The HA tag was cloned into the *Bam*HI/*Sac*I sites of pUCAP2 to create pUCAP3. DNA cloned into the MCS of pUCAP3 will have the HA tag at the C-terminus.

Specific oligos were designed to create an HA tag with a *Hind*lll overhang immediately before the initiation codon and a *Sal*I overhang immediately after the last codon of the tag. Direct oligo: HA5 (SEQ ID NO: 82). Reverse oligo: HA6 (SEQ ID NO: 83). The HA tag was created by annealing oligos (0.1 pg/uL) at 92 °C for 3 minutes and slowly bringing to room temperature. The HA tag was cloned into the *Hind*lll/*Sal*I site of pUCAP2 to create pUCAP4. DNA cloned into the MCS of pUCAP4 will have the HA tag at the N-terminus.

Specific primers were designed to add a 5'-*Asc*I site and a 3'-*Sph*I site flanking the *A. thaliana* IRT2 promoter to *Asc*I and *Sph*I of pUCAP1. Direct primer: IRT1 (SEQ ID NO: 86). Reverse primer: IRT2 (SEQ ID NO: 87). The IRT2 promoter was amplified from *Arabidopsis thaliana* using a 30 cycle Gradient PCR [(4 min 95 C, 30 sec 48-63 C, 2 min 68 C) with KTLA DNA polymerase under standard conditions]. The PCR product was digested with *AscI*/*Sph*I and *AscI*/*Sph*I cloned into of pUCAP1 giving pUCAP5.

pUCAP6 was created by replacing the Ubi3 promoter of pUCAP3 with the IRT2 promoter using the *AscI*/*Sph*I sites.

Generation of a vector containing the HA-tag for N-terminal fusions.

Oligonucleotides HA-tag-F (SEQ ID NO: 109) and HA-tag-R (SEQ ID NO: 110) were mixed and annealed using standard procedures. The annealed product generates compatible ends for *Hind*III and *Bam*HI restriction sites and was cloned into the plasmid vector pUC118, generating the plasmid pUC-HA.

Plant transformation vector containing the 5' UTR and 3' UTR regions of the *fad2* gene from *A. thaliana.*

A. thaliana genomic DNA was used as template and KTLA was the DNA polymerase of choice For PCR. Primers Fad5'UTR-F (SEQ ID NO: 111) and Fad5'UTR-R (SEQ ID NO: 112) were used to PCR amplify the 5' UTR, first intron and first codon of *fad2*, flanked by the restriction sites *Xho*I at the 5' end and *Nco*I, *Bam*HI at the 3' end. PCR reactions were performed under standard conditions as follow: 97 C for 30 sec, 35 cycles of amplification (45 sec at 94 C, 1 min at 55 C, 90 sec at 72 C) and a final extension of 5 min at 72 C. The PCR product was cloned into the plasmid vector pCR2.1 (Invitrogen).

Primers Fad3'UTR-F (SEQ ID NO: 113) and Fad3'UTR-R (SEQ ID NO: 114) were used to PCR amplify the 3' UTR of *fad2*. Reactions were performed as follow: 97 C for 10 sec and 35 cycles of amplification (30 sec at 94 C, 1 min at 60 C, 2.5 min at 72 C). The PCR product was cloned into the plasmid vector pCR2.1. The identities of both PCR products, *fad2* 5' UTR (SEQ ID NO: 44) and *Fad2* 3' UTR (SEQ ID NO: 45) were confirmed by DNA sequencing.

The plant transformation vector containing both *fad2* UTR regions was constructed in two steps: first, the *fad2* 5' UTR fragment was subcloned immediately downstream of the

CaMV35S promoter of a binary vector as a *XhoI/Bam*HI insert. Then, the *A. tumefaciens NOS* 3' UTR present in the plasmid, between the *Xba*I and *Hind*III restriction sites was replaced with the *A. thaliana fad2* 3'UTR fragment between the same sites, generating a plasmid called pFADUTR.

Cloning Hydroxylase and Bifunctional Hydroxylase genes into pUCAP3, pUCAP4 and pUCAP6

R. communis hydroxylase and *L. lindheimeri* bifunctional hydroxylase genomic clones were generated by PCR amplification using specific primers.

Ricinus communis hydroxylase with a C-terminal HA tag: Specific primers were designed to introduce a *Hind*lll site immediately upstream of the initiation codon and a *Bam*HI site immediately before stop codon. Direct primer: Castor 5'-HindIII (SEQ ID NO: 88). Reverse primer: Castor 3'-BamHI (SEQ ID NO: 89). The hydroxylase was amplified by PCR [5 cycles (4 min 94 C, 45 sec 94 C, 50 C 45 sec, 72 C) and then 25 cycles (45 sec 94 C, 45 sec 58 C, 2 min 72 °C) with KTLA under standard conditions]. The PCR product was digested with *Hind*III and *Bam*H1 and subsequently cloned into *Hind*III, *Bam*H1 of pUCAP3 expression vector giving *Rc*-pUCAP3.

Ricinus communis hydroxylase with a N-terminal HA tag: Direct primer: BamHI castor F (SEQ ID NO: 104). Reverse primer: castor XbaI MfeI R (SEQ ID NO: 98). The hydroxylase gene was amplified by PCR [5 thermal cycles (1 min, 92 C, 30 sec 50 C, 1.5 min 68 C) and then 25 thermal cycles (1 min, 92 C, 30 sec 57 C, 1.5 min 68 C) with KTLA DNA polymerase under standard conditions]. The PCR product was digested with *Bam*HI */Mfe*I and subcloned into the *Bam*HI/*Eco*RI sites of the pUC-HA vector.

Lesquerella lindheimeri bifunctional enzyme with an N-terminal HA tag: Specific primers were designed to introduce a *Sal*I site immediately upstream of the start codon and *Bam*H1 site immediately after the stop codon. Direct primer fend F SalI (SEQ ID NO: 90). Reverse primer: Fend R B-stop. (SEQ ID NO: 91). The bi-functional hydroxylase gene was amplified by PCR [5 cycles (4 min 94 C, 45 sec 94 C, 45 sec 50 C, 2 min 72 C) and then 25 cycles (45 sec 94 C, 45 sec 58 C, 2 min 72 C) with KTLA DNA polymerase under standard conditions]. The PCR product was digested with *Sal*I and *Bam*H1 subsequently cloned into *SalI/Bam*H1 of pUCAP4 giving *Rc*-pUCAP4.

L. gracilis bifunctional hydroxylase with a N-terminal HA tag: Direct primer: BamHI gracilis F (SEQ ID NO: 105). Reverse primer: gracilis XbaI MfeI R (SEQ ID NO: 101). The hydroxylase gene was amplified by PCR [5 thermal cycles (1 min, 92 C, 30 sec 50 C, 1.5 min 68 C) and then 25 thermal cycles (1 min, 92 C, 30 sec 57 C, 1.5 min 68 °C) with KTLA DNA polymerase under standard conditions]. The PCR product was digested with *Bam*HI/*Mfe*I and subcloned into the *Bam*HI / *Eco*RI sites of the pUC-HA vector.

The *Crepis biennis* and *Stokesia laevis* epoxygenase genes were subcloned as described above into the pUC-HA vector using BglII NcoI C. biennis F (SEQ ID NO: 108)/Crepis XbaI MfeI R (SEQ ID NO: 103) and BamHI NcoI S. epoxygenase F (SEQ ID NO: 106)/S.epoxygenase XbaI R (SEQ ID NO:107).

The *Crepis biennis* and *Stokesia laevis* epoxygenase genes lacking the HA sequence were subcloned as described above into a plant expression vector using Asc1 Nco1 C. biennis *F* (SEQ ID NO: 124)/Crepis XbaI MfeI R (SEQ ID NO: 103) and BamHI NcoI S. epoxygenase F (SEQ ID NO: 106)/S.epoxygenase XbaI R (SEQ ID NO:107).

Plant Expression Vectors

Constructs *Rc*-pUCAP3, *Ll*-pUCAP4, and *Rc*-pUCAP6 were digested with *Asc*I and *Pac*I to release the inserts and inserts were subsequently sub-cloned into the *Asc*I/*Pac*I sites of pBinPlusARS binary vector engineered as described by [Engelen et al. (1995) *Transgenic Res.* 4(4):288-290] giving *Rc*-3pBinPlusARS, *Ll*4-pBinPlusARS and *Rc*6-pBinPlusARS.

ΔKKGG castor, ΔT gracilis, *R. communis* hydroxylase, chimeric *fad2*/*R. communis* hydroxylase, *L. gracilis* bifunctional hydroxylase, chimeric *fad2*/*L. gracilis* bifunctional hydroxylase, *C. biennis* epoxygenase, and *S. laevis* epoxygenase genes were subcloned into a plant expression vector using *Nco*I/*Xba*I restriction enzyme sites. N-terminal HA tagged chimeric *fad2*/*R. communis* hydroxylase and N-terminal chimeric *fad2*/*R. communis* hydroxylase were removed from pUC-HA and subcloned into a plant expression vector using *Nco*I/*Xba*I restriction enzyme sites. The above constructs were also subcloned into a plant expression vector containing the *fad2* 5'UTR and *fad2* 3'UTR (pFADUTR), using the *Nco*I/*Xba*I restriction sites.

Example 17

This example describes the production of transgenic *Arabidopsis* plants, transgenic tomato callus, transgenic tomato hairy roots, *Arabidopsis* hairy root, soybean hairy root, and soybean composite plants using the plasmid vectors of Example 16.

Transformation of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*

Plant expression vectors harboring genes encoding hydroxylases, epoxygenases or chimeric *fad2* constructs were transformed into *Agrobacterium tumefaciens* LB4404 as follows. Agrobacterium was grown overnight in 100 mL of LB [(1% bacto tryptone, 0.5% sodium chloride and 0.5% bacto-yeast extract) supplemented with kanamycin (50 ug/mL), rifampicin (10 ug/mL), and streptomycin (150 ug/mL)]. 100 mL of LB supplemented in the same manner was inoculated with 1 mL of the overnight culture and grown at 30 C for 4 hrs. The culture was chilled for 10 minutes and cells were harvested by centrifugation. Cells were resuspended in 1 mL of ice cold $CaCl_2$ (20 mM) and dispensed into 100 µL aliquots. 1 µg of plasmid DNA was added to the cells, frozen on dry ice, put at 37 C for 5 minutes, and shaken for 90 minutes at 30 C in 1 mL LB. Cells were pelleted and resuspended in 100 µL of LB and plated on LB plates [(1% bacto tryptone, 0.5% sodium chloride, 0.5% bacto-yeast extract, and .15% agar) supplemented with kanamycin (50 ug/mL), rifampicin (10 ug/mL), and streptomycin (150 ug/mL)].

Transformation of *Agrobacterium rhizogenes* strain A4 was performed in the same manner as *Agrobacterium tumefaciens* strain LB4404 with the following exceptions: Media used was MGL [extract (2.5 g/L), tryptone (5 g/L), sodium chloride (5 g/L), L-glutamic acid (1 g/L), mannitol (5 g/L), potassium phosphate (0.26 g/L), magnesium sulfate heptahydrate (100 mg/L), and biotin (1 mg/L)] and MGL plates [yeast extract (2.5 g/L), tryptone (5 g/L), sodium chloride (5 g/L), L-glutamic acid (1 g/L), mannitol (5 g/L), potassium phosphate (0.26 g/L), magnesium sulfate heptahydrate (100 mg/L), biotin (1 mg/L), and bacto-agar (14 g/L)].

Plant Transformation

Arabidopsis thaliana was transformed via *Agrobacterium tumefaciens* following Clough and Bent [Clough & Bent (1998) *Plant J.* 19(3):249-257]. Briefly, 5 mL overnight

cultures of transformed LB4404 (LB-10ug/mL rifampicin, 50ug/mL kanamycin, 150µg/mL streptomycin) were grown at 30 °C. The 5 mL cultures were used to inoculate 500 mL LB (10 µg/mL rifampicin, 50 µg/mL kanamycin, 150 ug/mL streptomycin) and grown overnight at 30 C. Cultures were spun down (5K, 5min). Pellets were resuspended in 5% glucose + .02% Silwet L-77. The above ground parts of the plant were submerged into *Agrobacterium* solution for 5 min with gentle agitation. Plants were covered under a dome overnight.

Fatty Acid Analysis of *Arabidopsis thaliana* leaf and root tissue

Generation of plant material

Seed sterilization: Approximately 200 second generation seeds from transformed plants were placed in an eppendorf tube. 1mL of 20% bleach in ethanol was added and the tubes were left at room temperature for 15 minutes. The seeds were then washed 2X with 100% ethanol and opened tubes were left in the laminar flow hood to dry overnight.

Seed Germination: Approximately 50 seeds were placed on 0.5X MS plates, wrapped in parafilm, and kept at room temperature until germination.

Approximately 0.10g of root tissue or leaf tissue was put in a 1.5mL eppendorf tube and frozen on dry ice and subsequently ground with a pestle. The ground root tissue was then methylated with (500 µL 1% sodium methoxide in methanol), extracted with hexane, and trimethylsilylated (100 µL BSTAFA-TMCS, Supelco, 90 °C for 45 minutes). Samples were analyzed on an Agilent 6890 GC-5973 Mass Selective Detector (GC/MS) and an Agilent DB-23 capillary column (0.25 mm x 30 m x 0.25 um). The injector was held at 250 °C, the oven temperature was 235 °C, and a helium flow of 1.0 mL/min was maintained.

Table 14: Fatty Acid Analysis of extracts from *Arabidopsis thaliana* harboring a chimeric *fad2/R.communis* hydroxylase

Tissue	Construct	Line	%R	%L	%O
Leaves	4028*	6	1.19	15.82	1.87
Leaves	4028*	6	1.11	15.22	2.02
Roots	4028*	6	0.54	25.52	0.61
Roots	4028*	6	0.10	22.24	0.73
Roots	4062	3	1.44	21.18	5.09
Roots	3819	-	0	21.54	1.96

*Designates constructs with a HA tag on the N-terminus.

These GC/MS data indicate that a chimeric *fad2/R.communis* hydroxylase (4062 or 4028*) operably linked to 5' and 3' *fad2* UTRs was functional when expressed in *A. thaliana*. The percentages of ricinoleic acid listed in the table are a percentage of the total fatty acid. *A. thaliana* transformed with a vector containing no insert (3819), did not accumulate ricinoleic acid (R).

Hairy Root transformation protocol for tomato

Plant material preparation: This protocol can be used for tomato root transformation. Numerous strains of *A. rhizogenes* may be used as the transforming agent, however, strain A4 (ATCC number 43057) was used in this case. *Lycopersicon esculentum* cv. Rutgers, Money Maker or Mountain Spring, were used, although other varieties that are susceptible to *Meloidogyne incognita* (*M. incognita*) infection may be used. As a control, the resistant cultivar Motelle was used [Vos et al. (1998) *Nat. Biotechnol.* 16: 1365-1369]. This protocol can also be used to generate hairy root cultures from *Arabidopsis thaliana*, ecotype Columbia.

The transformation protocol is similar to that described previously [McCormick (1991) Transformation of tomato with *Agrobacterium tumefaciens*. in Plant Tissue Culture Manual, Fundamentals and Applications, K. Lindsey (ed), Kluwer, Vol. B6: 1-9]. Briefly, tomato seeds were sterilized with hypochlorite and grown in magenta boxes containing Gamborg's synthetic medium [Gamborg et al. (1968) *Exp. Cell Res.* 50:151-158] in daylight for 7 days, until cotyledons are completely unfolded. Cotyledons were removed sterilely and wounded in MSO medium (MS salts, 3% sucrose, Gamborg's B5 vitamins, pH 5.8) by removing both the proximal and distal tips with a razor blade. Wounded cotyledons were incubated for 1-2 days, adaxial side up, on filter paper placed on 150 mm^2 plates made with D1 medium (MS salts, 3% glucose, Gamborg's B5 vitamins, 1 mg/L zeatin, 0.8% Gel-rite agar). After this incubation period, cotyledons were cocultured with a suspension of *A. rhizogenes to* initiate transformation.

A. rhizogenes culture preparation: A glycerol stock of *A. rhizogenes* A4 was streaked onto MGL medium [McCormick (1991) Transformation of tomato with *Agrobacterium*

tumefaciens. in Plant Tissue Culture Manual, Fundamentals and Applications, K. Lindsey (ed), Kluwer, Volume B6: 1-9] and grown at 29 °C until individual colonies appeared. A single colony was used to inoculate a 15 mL culture of MGL medium, which was grown for one day in a shaking incubator at 29 °C, 100 rpm. On the following day, the bacteria were harvested by centrifugation at 3800 x g for 10 minutes. The resulting pellet was washed twice, without disturbing the pellet, with 15 mL of MSO medium and centrifuging at 3800 x g for 5 minutes. The final pellet was resuspended in 15 mL MSO medium and the optical density of the culture at 550 nm was determined. The density was adjusted to 0.4 with MSO medium. 10 mL of this culture was used for cocultivation after the addition of 50 µl of 0.074 M acetosyringone. Cocultivation was performed within one hour of the addition of acetosyringone.

Cocultivation of tomato cotyledons and *A. rhizogenes*: Onto each plate of cotyledons, 5 mL of *A. rhizogenes* culture was pipetted over the preincubated cotyledons using sterile technique. The plates were incubated at room temperature for 10 minutes, with occasional swirling of plates during this time. The bacterial suspension was then removed with a sterile pipette. The cotyledons were transferred gently, abaxial side up, using a scalpel or razor blade, to a new 100 x 20 mm Petri plate containing a Whatman filter paper disk on D1 medium. The plates were sealed with micropore tape and incubated for 2 days at room temperature near a south facing window.

Selection of transgenic roots: After cocultivation, the cotyledons were transferred, abaxial side up onto Gamborg's medium containing 200 mg/L cefotaxime at a density of 20-30 cotyledons per plate. The plates were sealed with micropore tape and incubated at room temperature in the dark for 10 days. On the 10^{th} day, the cotyledons were transferred to fresh selective media plate. After an additional 10 day period, hairy root initials were removed from the cotyledons using a sterile razor blade and incubated on selective medium with transfer to fresh plates after 10 days. To assess whether the hairy roots were cured of infection by *A. rhizogenes*, the roots were transferred to Gamborg's medium without cefotaxime and allowed to grow for 10 days. Any plates showing bacterial growth around the roots were discarded.

Root cultures were maintained on Gamborg's medium lacking selection by serial transfer every 20-30 days.

Fatty Acid Analysis of tomato hairy root extracts

Approximately 0.25g of root tissue was placed in a 1.5mL eppendorf tube and frozen on dry ice and subsequently ground with a pestle. The ground root tissue was then methylated with (500 µL 1% sodium methoxide in methanol), extracted with hexane, and trimethylsilylated (100 µL BSTAFA-TMCS, Supelco, 90 °C for 45 minutes). Samples were analyzed on an Agilent 6890 GC-5973 Mass Selective Detector (GC/MS) and an Agilent DB-23 capillary column (0.25 mm x 30 m x 0.25 um). The injector was held at 250 °C, the oven temperature was 235 °C, and a helium flow of 1.0 mL/min was maintained.

Table 15: Fatty Acid Analysis of tomato roots harboring a *R. communis* hydroxylase

Construct	Line	%R	%L	%O	Temp	Cultivar
4203	7	1.637	50.54	0.94	23	Money Maker
4203	7	1.17	50.48	1.20	23	Money Maker
4203	16	1.29	55.67	0.00	23	Money Maker
4203	16	1.07	52.04	1.89	23	Money Maker
4203	15	1.21	53.66	1.25	23	Money Maker
4203	15	0.91	51.57	1.63	23	Money Maker
3677	19	0	47.06	0.00	23	Money Maker

These GC/MS data indicate that a *R. communis* (4203) hydroxylase was functional when expressed in tomato hairy root tissue. The percentages of ricinoleic acid (%R) listed in the table are percentages of the total fatty acid. Tomato hairy roots transformed with a vector containing no insert (3677), did not accumulate ricinoleic acid (R). Linoleic and oleic acid percentages are listed under the columns %L and %O, respectively.

Table 16: Fatty Acid Analysis of tomato roots harboring a chimeric *fad2/R.communis* hydroxylase

Construct	Line	%R	%L	%O	Temp	Cultivar
3927	7	2.81	49.02	2.05	23	Rutgers
3927	7	1.97	51.78	2.22	23	Rutgers
3927	7	1.67	55	2.17	23	Rutgers
3927	20	1.03	52.38	1.04	15	Rutgers
3927	20	0.98	51.08	1.59	15	Rutgers
3927	20	0.75	50.89	1.14	23	Rutgers
3938*	14	1.02	47.92	1.25	23	Rutgers
3938*	14	0.973	48.57	2.25	23	Rutgers
3938*	18	0.49	49.45	1.45	23	Rutgers
3938*	18	0.86	47.98	2.16	23	Rutgers
3677		0	52.05	2.51	23	Rutgers

*Designates HA on N terminus

These GC/MS data indicate that a chimeric *fad2/R.communis* hydroxylase (3927 or 3938*) was functional when expressed in tomato hairy root. The percentages of ricinoleic acid (%R) listed in the table are percentages of the total fatty acid. Tomato hairy roots transformed with a vector containing no insert (3677) did not accumulate ricinoleic acid (R). Linoleic and oleic acid percentages are listed under the columns %L and %O, respectively.

Table 17: Chimeric *fad2/R.communis* hydroxylase with 5' and 3' *fad2* UTRs

Construct	Line	%R	%L	%O	Temp	Cultivar
4062	19	1.26	48.04	6.99	23	Rutgers
4062	19	2.25	48.22	4.59	23	Rutgers
4062	19	1.97	50.19	3.60	23	Rutgers
4028*	12	2.38	50.54	2.43	15	Rutgers
4028*	12	2.36	52.64	2.70	15	Rutgers
4028*	12	1.13	51.34	4.19	23	Rutgers
3677	2	0	53.32	0.84	RT	Rutgers
4028*	5	0.95	53.15	2.49	RT	Mountain Spring
4028*	5	1.3	54.8	1.55	RT	Mountain Spring
4028*	5	0.58	47.61	2.56	RT	Mountain Spring
3677	2	0	57.94	0.87	RT	Mountain Spring

*Designates HA on N terminus. RT = room temperature

These GC/MS data indicate that a chimeric *Fad2/R.communis* hydroxylase (4062 or 4028*) operably linked to 5' and 3' *fad2* UTRs was functional when expressed in tomato hairy root. The percentages of ricinoleic acid listed in the table are percentages of the total fatty acid. Tomato hairy roots transformed with a vector containing no insert (3677) did not accumulate ricinoleic acid (R).

Hairy Root Transformation Protocol for soybean

Seed sterilization: Approximately 250 seeds were placed in a 100 X 25 mm plate and placed in a desicator in a fume hood. Using a 350 mL beaker, 2 mL of concentrated HCl was carefully added to 200 mL of 100% bleach and the beaker was placed inside the desicator to expose the seeds to sterilizing gas. After 24 hours, the procedure was repeated. This was done 3 times for a total of 3 sterilizations. To test for sterility, 10 seeds were placed in LB and put in a shaker at 37 °C for 24 hour. If the LB was clear, indicating no bacterial growth, the seeds were sealed in the Petri dish and germinated at a later date. If there was bacterial growth, the sterilization procedure was performed again.

Seed Germination: 9 seeds were placed on 0.25X solid MS plates, wrapped in parafilm, and kept at room temperature for 7 days.

A. rhizogenes culture preparation: A glycerol stock of *A. rhizogenes* A4 was streaked onto MGL medium [McCormick (1991) Transformation of tomato with *Agrobacterium tumefaciens*. in Plant Tissue Culture Manual, Fundamentals and Applications, K. Lindsey (ed), Kluwer, Volume B6: 1-9] and grown at 29 °C until individual colonies appeared. A single colony was used to inoculate a 15 mL culture of LB + Kanamycin medium, which was grown for one day in a shaking incubator at 29 °C, 100 rpm. On the following day, the bacteria were harvested by centrifugation at 3800 x g for 10 minutes. The resulting pellet was resuspended in MSO to a final optical density of 0.2-0.3. Acetosyringone was then added to a final concentration of 375um. Cocultivation was performed within one hour of the addition of acetosyringone.

Explant Excision: The cotyledons were cut from the main axis making sure that the axillary bud was removed.

Cocultivation of soybean cotyledons and *A. rhizogenes*: Soybean cotyledons were added to the culture using sterile technique. The cultures were then vacuum infiltrated for 2 minutes and incubated at room temperature for 20 minutes. The bacterial suspension was then removed with a sterile pipette. The cotyledons were transferred gently, abaxial side up, using tweezers, to a 100 x 20 mm Petri plate containing a Whatman filter paper disk soaked in MSO. The plates were sealed with micropore tape and incubated for 2 days at room temperature near a south facing window.

Selection of transgenic roots: After cocultivation, the cotyledons were transferred, abaxial side up onto MS solid medium containing 500 mg/L carbenicillin at a density of 10 cotyledons per plate. The plates were sealed with micropore tape and incubated at room temperature. About 28 days post-inoculation, hairy roots were removed from the cotyledons using a sterile razor blade and incubated on Gamborgs medium plus selection.

Hairy Root Transformation Protocol for *Arabidopsis thaliana*

Seed sterilization: Approximately 200 seeds were placed in an eppendorf tube. 1mL of 20% bleach in ethanol was added and the tubes were left at room temperature for 15

minutes. The seeds were then washed 2X with 100% ethanol and opened tubes were left in the laminar flow hood to dry overnight.

Seed Germination: Approximately 50 seeds were placed on 0.5X solid MS plates, wrapped in parafilm, and kept at room temperature until germination.

A. rhizogenes culture preparation: A glycerol stock of *A. rhizogenes* A4 was streaked onto MGL medium [McCormick (1991) Transformation of tomato with *Agrobacterium tumefaciens*. in Plant Tissue Culture Manual, Fundamentals and Applications, K. Lindsey (ed), Kluwer, Volume B6: 1-9] and grown at 29 °C until individual colonies appeared. A single colony was used to inoculate a 15 mL culture of LB + Kanamycin medium, which was grown for one day in a shaking incubator at 29 °C, 100 rpm. On the following day, the bacteria were harvested by centrifugation at 3800 x g for 10 minutes. The resulting pellet was resuspended in MSO to a final optical density of 0.2-0.3. Acetosyringone was then added to a final concentration of 375um. Cocultivation was performed within one hour of the addition of acetosyringone.

Explant Excision: *A. thaliana* cotyledons were removed sterilely and wounded in MSO medium (MS salts, 3% sucrose, Gamborg's B5 vitamins, pH 5.8) by removing both the proximal and distal tips with a razor blade. Wounded cotyledons were incubated for 1-2 days, adaxial side up, on filter paper placed on 150 mm^2 plates made with D1 medium (MS salts, 3% glucose, Gamborg's B5 vitamins, 1 mg/L zeatin, 0.8% Gel-rite agar). After this incubation period, cotyledons were cocultured with a suspension of *A. rhizogenes to* initiate transformation.

Cocultivation of *A. thaliana* cotyledons and *A. rhizogenes*: *A. thaliana* cotyledons were added to the *A. rhizogenes* culture using sterile technique and left at room temperature for 10 minutes. The bacterial suspension was then removed with a sterile pipette. The cotyledons were transferred gently, abaxial side up, using a sterile spatula, to a Whatman filter paper disk in a 100 x 20 mm Petri plate containing solid Gamborgs medium plus 500mg/L carbenicillin. The plates were sealed with micropore tape and incubated for at room temperature near a south facing window.

Selection of transgenic roots: About 10 days post-inoculation, hairy roots were removed from the cotyledons using a sterile razor blade and placed on Gamborgs medium plus selection.

Callus Transformation Protocol

Plant material preparation: This protocol can be used to generate transgenic tomato callus. All transformations carried out used *Agrobacterium tumefaciens* strain LB4404 and the tomato cultivar *Lycopersicon esculentum* cv. Rutgers, Money Maker, or Mountain Spring. Tomato cotyledons were grown as described in the hairy root transformation section.

A. tumefaciens culture preparation: A glycerol stock of *A. tumefaciens* LB4404 was streaked onto LB medium (rifampicin 10 mg/L, streptomycin 150 mg/L, kanamycin 50 mg/L) (McCormick, 1991) and grown at 29 °C until individual colonies appeared. A single colony was used to inoculate a 15 mL culture of LB medium, which was grown for one day in a shaking incubator at 29 °C, 100 rpm. On the following day, the bacteria were harvested by centrifugation at 3800 x g for 10 minutes. The resulting pellet was washed twice, without disturbing the pellet, with 15 mL of MSO medium and centrifuging at 3800 x g for 5 minutes. The final pellet was resuspended in 15 mL MSO medium and the optical density of the culture at 550 nm was determined. The density was adjusted to 0.4 with MSO medium. 10 mL of this culture was used for cocultivation after the addition of 50 µL of 0.074 M acetosyringone. Cocultivation was performed within one hour of the addition of acetosyringone.

Cocultivation of tomato cotyledons and *A. tumefaciens*: Cocultivation was carried out as described in the hairy root transformation section with the exception of using *A. tumefaciens*.

Selection of transgenic callus: After cocultivation, the cotyledons were transferred, abaxial side up onto 2Z medium (4.3 g MS salt/L, 20% sucrose, 1 mg zeatin/L, 100 mg/L inositol, 1X Nitsch vitamin, 1X folic acid, 8 g/L tissue culture agar) containing 200 mg/L cefotaxime and 100 mg/L kanamycin at a density of 20-30 cotyledons per plate. The plates were sealed with micropore tape and incubated at room temperature in the dark for 10 days. Every 10 days, the cotyledons were transferred to fresh selective media plate. Explants started to grow green or white callus after two to three weeks. Explants that were dying (turning brown) were removed. Callus was excised from explants that contained dying tissue. The callus was maintained on Gamborg's medium.

Composite plant protocol for soybean:

Agrobacterium rhizogenes A4 cultures were grown overnight at 30°C in Luria Broth with the appropriate antibiotics. Cultures were spun down at 4,000g for 10 minutes. Cells were suspended with ¼ MS to a final $O.D._{600nm}$ between 0.2-0.5.

Sterile soybean seeds (Cl_2 gas treated seeds) were planted in soil. Young shoots lacking any inflorescences were cut in the middle of the internode region. Shoots were transplanted into one cm^2 FibrGro® cubes. Each transplant was inoculated with 4 mL of suspended *A. rhizogenes*, placed in a flat, covered with a clear lid, and left on the bench top for one day to allow for acclimation. On the second day the lid was removed to let the cubes dry out. Transplants were then watered and covered. Roots appeared between two and four weeks. Transformed roots can be identified by a visible marker. The untransformed roots should be excised. After several weeks, shoots can be transplanted to sand for nematode infection assays.

Example 18

This example describes assays to measure anthelmintic activity of transgenic plants.

Infection of hairy roots: Plates for assays were prepared by transferring one growing hairy root tip, 1-2 cm long, from a stock root plate onto 100 x 15 cm Petri dishes containing approximately 30 mL of Gamborg's media in which the Gel-rite agar had been replaced by 3.0% Phytagel (Sigma catalog P-8169). At least two plates were used per transgenic line per assay. As a control, we used a hairy root line that was generated using *A. rhizogenes* that had been transformed with a plant transformation plasmid that does not carry any coding sequence after the promoter. Assay plates were sealed with micropore tape and incubated at 28 °C for 4-7 days prior to infection with *Meloidogyne incognita* eggs.

Preparation of *Meloidogyne incognita* inoculum: *M. incognita* eggs were harvested from a greenhouse-grown tomato plant (*Lycopersicon esculentum* cv. Mountain Spring) that had been infected 28-42 days previously with 5000 *M. incognita* eggs using a protocol described previously [Hussey & Barker (1973) *Plant Disease Reporter* 57:1025-1028]. Aerial tissues of the tomato plant were removed and the root mass was freed from soil by gentle agitation in a bucket filled with tap water. The root mass was transferred to a

household blender with the addition of 500 mL 10% bleach solution (Clorox bleach in tap water) and chopped into fine pieces using the puree setting. The root slurry was transferred to a 200 mesh sieve seated on top of a 500 mesh sieve (VWR catalog numbers 57334-480 and 57334-492, respectively) and eggs were collected on the 500 mesh sieve by rinsing vigorously with tap water. Eggs were further cleaned and concentrated by sucrose density centrifugation. Eggs were collected in approximately 30 mL of water and were pipetted on top of 30 mL of 30% sucrose solution in a 50 mL centrifuge tube and banded by centrifugation in a swinging bucket rotor at 1000 x g for 10 minutes. The eggs were collected using a Pasteur pipette and rinsed extensively to remove sucrose on a small 500 mesh sieve using tap water. Eggs were collected in a small amount of water and stored at 4°C until use.

Sterilization of inoculum: Approximately 100,000 stored *M. incognita* eggs were placed in a 15 mL centrifuge tube and brought to 10 mL volume with a 10% bleach solution. The tube was agitated for 5 minutes and eggs were collected by centrifugation as described above. The supernatant was removed and the eggs were rinsed 3 times with sterile water. Eggs were resuspended in 1mL of water and counted using a McMaster worm egg counting chamber. Only eggs containing vermiform larvae were counted.

Alternatively, if hatched J2 larvae were to be used as inoculum, eggs were hatched using a standard protocol. Larvae were collected by centrifugation as above and sterilized as described in Atkins, 1996 [Atkinson et al. (1996) *J. Nematol.* 28:209-215], using sequential incubations in penicillin, streptomycin sulfate, and chlorhexidine solutions, followed by rinsing in sterile water.

Inoculation and monitoring of assay: Hairy root infections were initiated by adding either 300 eggs or 100 J2 larvae per plate in 10μL, using sterile technique. Plates were resealed with parafilm after inoculum addition and monitored at 2, 7, 14, 21, 28 and 35 days. Plates that showed contamination with bacteria or fungi were discarded. Nematode-induced infection galls were visible under low-power magnification at 7 days, and adult females were visible at 25-30 days.

Scoring of infection assays

Gall number: The number of galls per plate was determined after 30-35 days by counting under low-power magnification. Total number of galls, as well as the number of adult and gravid females, was recorded. Alternatively, total number of *M. incognita* at all stages was determined by fuchsin staining of the roots [Eisenback (2000) Techniques for measuring nematode development and egg production. in Laboratory Techniques in Nematode Ecology. Wheeler et al., eds. Society of Nematologists: Hyattsville, MD. p.1-4].

Brood size: Gravid females were excised from each separate assay plate and placed in microcentrifuge tubes. 1 mL of 10% bleach was added to each tube and the tubes were agitated for 3 minutes. Freed eggs were collected by microcentrifugation (1000 x g, 2 minutes), rinsed three times with sterile water, and counted as described above. Brood size was recorded as eggs/female.

Brood viability: After counting, eggs from individual plates were transferred in 500 µL water to wells of a 24-well plate and incubated at room temperature in the dark for 7 days. The number of newly hatched J2 larvae visible after this period was determined and recorded. Ability of eggs or larvae to re-infect hairy roots was determined by inoculating control roots with eggs or J2's as described.

Scoring system based on root galling: A relatively higher throughput scoring system can be utilized when the number of plates becomes difficult to score by the methods listed above. The following table is an example of a rating system based on visual estimation of root damaged caused by *Meloidogyne spp*:

Damage Score	Description
0	No galls
1	1-2 small galls
3	3-5 small galls
5	>5 small galls, but no multiple galls
10	Several small galls and at least one multiple gall
25	About 25% of the roots with multiple galls; many small galls
50	About 50% of the roots with multiple galls
75	About 75% of the roots with multiple galls
90	Entire root system is galled and stunted

Soybean cyst nematode pot assay

This assay is used to evaluate the resistance of soybean plants to infection by and reproduction of the soybean cyst nematode (*Heterodera glycines*) on roots. Three or four inch diameter square pots were filled with clean sand and watered thoroughly. Soybean seeds, or alternatively any rooted plant parts, were planted one per pot in the center of the pot and watered well to remove air pockets. The pots were incubated in the greenhouse or growth chamber at 20 °C to 30 °C until the plants reached a suitable age for inoculation. Soybeans started from seed were typically inoculated 2-3 weeks after planting, while transplants were inoculated 1-3 days after planting. The test inoculum consisted of eggs from ripe *H. glycines* cysts collected from the soil and roots of infested soybean plants. A 250 micron mesh sieve was used to collect the cysts, which were then crushed in a Tenbroeck glass tissue homogenizer to release the eggs. The eggs were further purified by sieving and centrifugation over 40% sucrose solution at 4000 RPM for 5 minutes. Inoculum for an experiment consisted of water containing 500 vermiform eggs per mL. Five mL of the egg suspension was pipetted over the surface of the sand containing the test plants and the eggs were lightly watered in. The test plants were then returned to the greenhouse or growth chamber and incubated for 3-4 weeks to allow for root infection and cyst formation. The roots were then harvested by gently removing the pot and sand and rinsing in water. The severity of nematode infection was measured by counting the number of white nematode cysts adhering to the root system. Alternatively, the sand and roots could be diluted in water and passed over a 250 micron sieve to collect and concentrate the cysts for storage or counting.

Use of tomato hairy roots for assay of cyst nematode infections: The assay described above can also be used to determine the ability of cyst nematode to infect tomato roots using the cyst nematode strain TN2.

Example 19

Table 18: Sequence ID numbers for hydroxylase and epoxygenase genes

Construct	cDNA	Amino acid
Ricinus communis	SEQ ID NO: 1	SEQ ID NO: 13
Lesquerella fendleri	SEQ ID NO: 2	SEQ ID NO: 14
Lesquerella lindheimeri	SEQ ID NO: 3	SEQ ID NO: 15
Lesquerella gracilis A	SEQ ID NO: 4	SEQ ID NO: 16
Lesquerella gracilis B	SEQ ID NO: 5	SEQ ID NO: 17
Crepis biennis	SEQ ID NO: 6	SEQ ID NO: 18
fad2/R. communis	SEQ ID NO: 7	SEQ ID NO: 19
fad2/L. fendleri	SEQ ID NO: 8	SEQ ID NO: 20
fad2/L. lindheimeri	SEQ ID NO: 9	SEQ ID NO: 21
fad2/L. gracilis A	SEQ ID NO: 10	SEQ ID NO: 22
fad2/L. gracilis B	SEQ ID NO: 11	SEQ ID NO: 23
fad2/C. biennis	SEQ ID NO: 12	SEQ ID NO: 24
R. communis ΔKKGG	SEQ ID NO: 25	SEQ ID NO: 34
L. gracilis B ΔT	SEQ ID NO: 26	SEQ ID NO: 35
Stokesia laevis	SEQ ID NO: 27	SEQ ID NO: 36
R. communis optimization 2	SEQ ID NO: 28	SEQ ID NO: 37
S. laevis optimization 2	SEQ ID NO: 29	SEQ ID NO: 38
R. communis optimization 1	SEQ ID NO: 30	SEQ ID NO: 39
L. gracilis B optimization	SEQ ID NO: 31	SEQ ID NO: 40
C. biennis optimization	SEQ ID NO: 32	SEQ ID NO: 41
S. laevis optimization 1	SEQ ID NO: 33	SEQ ID NO: 42

Arabidopsis thaliana FAD2 5'-untranslated region (SEQ ID NO: 43 and 44) and *Arabidopsis thaliana* FAD2 3'-untranslated region (SEQ ID NO: 45).

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.